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GENETIC TOXICOLOGY

SALMONELLA TYPHIMURIUM MUTAGENICITY TEST PROTOCOL

Testing was performed as reported by Zeiger *et al.* (1992). Sodium nitrite was sent to the laboratory as a coded aliquot from Radian Corporation (Austin, TX). It was incubated with the *Salmonella typhimurium* tester strains TA98 and TA100 either in buffer or S9 mix (metabolic activation enzymes and cofactors from Aroclor 1254-induced male Sprague-Dawley rat or Syrian hamster liver) for 20 minutes at 37° C. Top agar supplemented with L-histidine and d-biotin was added, and the contents of the tubes were mixed and poured onto the surfaces of minimal glucose agar plates. Histidine-independent mutant colonies arising on these plates were counted following incubation for 2 days at 37° C.

Each trial consisted of triplicate plates of concurrent positive and negative controls and five doses of sodium nitrite. The high dose was limited to 10,000 µg/plate. All positive trials were repeated under the conditions that elicited the positive response.

In this assay, a positive response is defined as a reproducible, dose-related increase in histidine-independent (revertant) colonies in any one strain/activation combination. An equivocal response is defined as an increase in revertants that is not dose related, is not reproducible, or is not of sufficient magnitude to support a determination of mutagenicity. A negative response is obtained when no increase in revertant colonies is observed following chemical treatment. There is no minimum percentage or fold increase required for a chemical to be judged positive or weakly positive.

RAT AND MOUSE BONE MARROW MICRONUCLEUS TEST PROTOCOL

The standard three-exposure protocol is described in detail by Shelby *et al.* (1993). Male F344/N rats and B6C3F$_1$ mice were injected intraperitoneally (three times at 24-hour intervals) with sodium nitrite dissolved in phosphate-buffered saline. Solvent control animals were injected with phosphate-buffered saline only. The positive control animals received injections of cyclophosphamide. The animals were killed 24 hours after the third injection, and blood smears were prepared from bone marrow cells obtained from the femurs. Air-dried smears were fixed and stained; 2,000 polychromatic erythrocytes (PCEs) were scored for the frequency of micronucleated cells in up to five animals per dose group.

The results were tabulated as the mean of the pooled results from all animals within a treatment group plus or minus the standard error of the mean. The frequency of micronucleated cells among PCEs was analyzed by a statistical software package that tested for increasing trend over dose groups with a one-tailed Cochran-Armitage trend test, followed by pairwise comparisons between each dosed group and the control group (ILS, 1990). In the presence of excess binomial variation, as detected by a binomial dispersion test, the binomial variance of the Cochran-Armitage test was adjusted upward in proportion to the excess variation. In the micronucleus test, an individual trial is considered positive if the trend test P value is less than or equal to 0.025 or if the P value for any single dosed group is less than or equal to 0.025 divided by the number of dosed groups. A final call of positive for micronucleus induction is preferably based on reproducibly positive trials (as noted above). Ultimately, the final call is determined by the scientific staff after considering the results of statistical analyses, the reproducibility of any effects observed, and the magnitudes of those effects.

MOUSE PERIPHERAL BLOOD MICRONUCLEUS TEST PROTOCOL

A detailed discussion of this assay is presented by MacGregor *et al.* (1990). At the end of the 14-week toxicity study, peripheral blood samples were obtained from male and female mice. Smears were immediately prepared and fixed in absolute methanol. The methanol-fixed slides were stained with acridine orange and coded. Slides were scanned to determine the frequency of micronuclei in 2,000 normochromatic erythrocytes (NCEs) in each of 10 animals per exposure group.

The results for NCEs in mouse peripheral blood were tabulated as described for PCEs in the rat and mouse bone marrow micronucleus test protocol.

EVALUATION PROTOCOL

These are the basic guidelines for arriving at an overall assay result for assays performed by the National Toxicology Program. Statistical as well as biological factors are considered. For an individual assay, the statistical procedures for data analysis have been described in the preceding protocols. There have been instances, however, in which multiple aliquots of a chemical were tested in the same assay, and differing results were obtained among aliquots and/or among laboratories. Results from more than one aliquot or from more than one laboratory are not simply combined into an overall result. Rather, all the data are critically evaluated, particularly with regard to pertinent protocol variations, in determining the weight of evidence for an overall conclusion of chemical activity in an assay. In addition to multiple aliquots, the *in vitro* assays have another variable that must be considered in arriving at an overall test result. *In vitro* assays are conducted with and without exogenous metabolic activation. Results obtained in the absence of activation are not combined with results obtained in the presence of activation; each testing condition is evaluated separately. The summary table in the Abstract of this Technical Report presents a result that represents a scientific judgement of the overall evidence for activity of the chemical in an assay.

RESULTS

Sodium nitrite (100-10,000 µg/plate) was mutagenic in *S. typhimurium* strain TA100, with and without Aroclor 1254-induced hamster and rat liver S9 enzymes; no mutagenicity was observed in strain TA98 (Table E1; Zeiger *et al.*, 1992). When sodium nitrite was administered by intraperitoneal injection at 6.25 to 200 mg/kg to male rats three times at 24-hour intervals, no significant increase in the frequency of micronucleated PCEs was observed in any of the dose groups (Table E2). The initial trial was judged to be positive, based on the trend test (P=0.001); however, results of a repeat trial, in which 50 mg/kg was the highest nonlethal dose tested, were negative, and the rat bone marrow micronucleus test with sodium nitrite was judged to be negative overall. A similar study in which male mice were administered 7.81 to 250 mg/kg also gave negative results (Table E3). A third *in vivo* study, a peripheral blood micronucleus test in mice administered sodium nitrite (375-5,000 ppm) for 14 weeks, showed no significant increase in the frequency of micronucleated NCEs in either males or females (Table E4). Thus, sodium nitrite demonstrated mutagenic activity in a strain of *S. typhimurium* that mutates via base-pair substitution, but no indication of chromosomal damage was observed in three micronucleus studies conducted in rats and mice *in vivo*.

TABLE E1
Mutagenicity of Sodium Nitrite in *Salmonella typhimurium*[a]

Strain	Dose (µg/plate)	Revertants/Plate[b]					
		-S9		+30% hamster S9		+30% rat S9	
		Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
TA100	0	139 ± 19.4	171 ± 5.5	150 ± 0.7	158 ± 12.0	160 ± 0.6	173 ± 6.2
	100	143 ± 14.1		148 ± 8.5	187 ± 5.6	201 ± 13.3	199 ± 14.7
	333	126 ± 8.7		165 ± 4.4	182 ± 13.5	197 ± 9.2	233 ± 6.4
	1,000	142 ± 7.5	211 ± 19.9	223 ± 4.6	220 ± 4.7	237 ± 12.5	230 ± 11.8
	1,666		224 ± 10.6				
	3,333	195 ± 6.9	274 ± 21.4	341 ± 6.4	309 ± 11.7	259 ± 36.8	312 ± 10.4
	6,666		376 ± 7.5				
	10,000	280 ± 9.9	443 ± 12.0	508 ± 29.5	451 ± 20.1	390 ± 38.8	353 ± 5.4
Trial summary		Positive	Positive	Positive	Positive	Weakly Positive	Positive
Positive control[c]		1,036 ± 10.2	878 ± 22.7	832 ± 34.1	894 ± 24.1	568 ± 22.5	555 ± 18.3
TA98	0	31 ± 0.9		42 ± 1.7		40 ± 4.8	
	100	27 ± 0.6		40 ± 4.6		33 ± 3.6	
	333	26 ± 4.2		39 ± 3.1		41 ± 3.8	
	1,000	30 ± 3.5		47 ± 4.2		29 ± 1.0	
	3,333	25 ± 2.6		42 ± 2.1		33 ± 2.8	
	10,000	26 ± 2.8		30 ± 5.2		21 ± 5.0	
Trial summary		Negative		Negative		Negative	
Positive control		432 ± 19.0		539 ± 6.1		155 ± 14.3	

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[a] Study was performed at SRI International. The detailed protocol and these data are presented by Zeiger *et al.* (1992). 0 µg/plate was the solvent control.

[b] Revertants are presented as mean ± standard error from three plates.

[c] The positive controls in the absence of metabolic activation were sodium azide (TA100) and 4-nitro-*o*-phenylenediamine (TA98). The positive control for metabolic activation with both strains was 2-aminoanthracene.

TABLE E2
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Rats Treated with Sodium Nitrite by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Rats with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]
Trial 1				
Phosphate-buffered saline[d]	0	5	1.10 ± 0.24	
Cyclophosphamide[e]	25	5	26.20 ± 2.95	0.0000
Sodium nitrite	6.25	5	0.90 ± 0.33	0.6411
	12.5	5	0.80 ± 0.30	0.7109
	25	5	1.10 ± 0.48	0.5000
	50	5	1.30 ± 0.60	0.3707
	100	5	2.70 ± 0.60	0.0180
	200	Lethal		
			P=0.001[f]	
Trial 2				
Phosphate-buffered saline	0	5	1.10 ± 0.51	
Cyclophosphamide	25	3	24.83 ± 2.52	0.0000
Sodium nitrite	25	5	1.00 ± 0.45	0.5635
	50	5	1.10 ± 0.37	0.5000
			P=0.500	

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[a] Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the solvent control; significant at $P \leq 0.005$ for trial 1 and at $P \leq 0.012$ for trial 2 (ILS, 1990)

[d] Solvent control

[e] Positive control

[f] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at $P \leq 0.025$ (ILS, 1990)

TABLE E3
Induction of Micronuclei in Bone Marrow Polychromatic Erythrocytes of Male Mice Treated with Sodium Nitrite by Intraperitoneal Injection[a]

Compound	Dose (mg/kg)	Number of Mice with Erythrocytes Scored	Micronucleated PCEs/ 1,000 PCEs[b]	P Value[c]
Phosphate-buffered saline[d]	0	5	1.10 ± 0.51	
Cyclophosphamide[e]	50	5	28.60 ± 4.24	0.0000
Sodium nitrite	7.81	5	1.80 ± 0.34	0.1933
	15.63	5	1.80 ± 0.60	0.1933
	31.25	5	1.60 ± 0.78	0.2608
	62.5	5	1.60 ± 0.73	0.2608
	125	5	1.80 ± 0.49	0.1933
	250	Lethal		
			P=0.343[f]	

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[a] Study was performed at Integrated Laboratory Systems, Inc. The detailed protocol is presented by Shelby *et al.* (1993). PCE=polychromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the solvent control; significant at $P \leq 0.005$ (ILS, 1990)

[d] Solvent control

[e] Positive control

[f] Significance of micronucleated PCEs/1,000 PCEs tested by the one-tailed trend test; significant at $P \leq 0.025$ (ILS, 1990)

TABLE E4
Frequency of Micronuclei in Peripheral Blood Erythrocytes of Mice Following Administration of Sodium Nitrite in Drinking Water for 14 Weeks[a]

	Concentration (ppm)	Number of Mice with Erythrocytes Scored	Micronucleated NCEs/ 1,000 NCEs[b]	P Value[c]
Male				
	0	10	0.65 ± 0.17	
	375	10	0.80 ± 0.15	0.2887
	750	10	0.50 ± 0.13	0.7343
	1,500	10	0.85 ± 0.22	0.2325
	3,000	10	0.90 ± 0.12	0.1845
	5,000	10	0.80 ± 0.08	0.2887
			P=0.204[d]	
Female				
	0	10	0.50 ± 0.17	
	375	10	0.70 ± 0.20	0.2070
	750	10	0.55 ± 0.16	0.4136
	1,500	10	0.55 ± 0.14	0.4136
	3,000	10	0.60 ± 0.16	0.3349
	5,000	10	0.45 ± 0.12	0.5907
			P=0.704	

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[a] Study was performed at SITEK Research Laboratories, Inc. The detailed protocol is presented by MacGregor *et al.* (1990). NCE=normochromatic erythrocyte

[b] Mean ± standard error

[c] Pairwise comparison with the control; significant at $P \leq 0.005$ (ILS, 1990)

[d] Significance of micronucleated NCEs/1,000 NCEs tested by the one-tailed trend test; significant at $P \leq 0.025$ (ILS, 1990)

APPENDIX F
CLINICAL PATHOLOGY RESULTS

TABLE F1 Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite 222
TABLE F2 Hemoglobin, Methemoglobin, and Serum and Gastric Nitrosamine Data for Rats on Days 70 and 71 in the 14-Week Drinking Water Study of Sodium Nitrite 228

Table F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Male						
Hematology						
n						
Day 5	9	6	10	7	8	9
Day 19	10	10	9	10	10	9
Week 14	10	10	10	9	10	10
Hematocrit (%)						
Day 5	41.6 ± 0.5[b]	41.1 ± 0.6[c]	39.2 ± 0.6*	40.0 ± 0.4[d]	41.0 ± 0.7[b]	39.5 ± 0.6[b]
Day 19	43.5 ± 0.2	43.9 ± 0.4	44.3 ± 0.4[b]	42.8 ± 1.0	46.3 ± 1.1	37.7 ± 1.3[b]
Week 14	43.8 ± 0.6	44.1 ± 0.5	42.0 ± 0.6	42.2 ± 0.6	45.5 ± 0.5	46.5 ± 0.7*
Hemoglobin (g/dL)						
Day 5	14.5 ± 0.2[b]	14.2 ± 0.2[c]	13.6 ± 0.2*	13.8 ± 0.1[d]	14.2 ± 0.2[b]	13.8 ± 0.2[b]
Day 19	14.9 ± 0.1	15.0 ± 0.1	15.1 ± 0.1[b]	14.7 ± 0.3	15.3 ± 0.3	12.5 ± 0.4**[b]
Week 14	15.0 ± 0.2	15.2 ± 0.1	14.7 ± 0.2	14.7 ± 0.2	15.7 ± 0.1*	16.0 ± 0.3**
Erythrocytes ($10^6/\mu L$)						
Day 5	7.08 ± 0.08[b]	6.95 ± 0.13[c]	6.90 ± 0.10	6.79 ± 0.08[d]	6.94 ± 0.13[b]	6.89 ± 0.12[b]
Day 19	7.61 ± 0.05	7.65 ± 0.10	7.90 ± 0.08*[b]	7.54 ± 0.17	8.44 ± 0.15**	8.10 ± 0.21**[b]
Week 14	8.30 ± 0.12	8.39 ± 0.08	7.97 ± 0.13	7.91 ± 0.10	8.29 ± 0.08	8.92 ± 0.25
Reticulocytes ($10^6/\mu L$)						
Day 5	0.45 ± 0.03	0.47 ± 0.05	0.72 ± 0.05**	0.51 ± 0.05	0.67 ± 0.06**	0.69 ± 0.03**[d]
Day 19	0.21 ± 0.02	0.19 ± 0.01	0.21 ± 0.02	0.23 ± 0.03	0.37 ± 0.02**	0.58 ± 0.05**
Week 14	0.22 ± 0.02	0.22 ± 0.03	0.26 ± 0.02	0.18 ± 0.02	0.30 ± 0.03*	0.37 ± 0.04**
Nucleated erythrocytes ($10^3/\mu L$)						
Day 5	1.00 ± 0.33	3.00 ± 0.45	2.80 ± 0.65	1.57 ± 0.69	2.75 ± 1.03	2.78 ± 0.49
Day 19	0.60 ± 0.22	0.90 ± 0.23	0.89 ± 0.35	1.20 ± 0.44	2.70 ± 0.52**	5.33 ± 1.12**
Week 14	0.70 ± 0.30	0.70 ± 0.21	0.60 ± 0.27	0.89 ± 0.20	1.10 ± 0.41	1.70 ± 0.50
Mean cell volume (fL)						
Day 5	58.8 ± 0.3[b]	59.2 ± 0.3[c]	56.9 ± 0.2**	59.0 ± 0.4[d]	59.1 ± 0.4[b]	57.4 ± 0.3[b]
Day 19	57.1 ± 0.4	57.4 ± 0.5	56.2 ± 0.5[b]	56.9 ± 0.5	54.9 ± 0.9	46.4 ± 0.5**[b]
Week 14	52.8 ± 0.2	52.5 ± 0.2	52.8 ± 0.1	53.3 ± 0.3	55.0 ± 0.2**	52.4 ± 1.2**
Mean cell hemoglobin (pg)						
Day 5	20.4 ± 0.1[b]	20.5 ± 0.1[c]	19.8 ± 0.1*	20.3 ± 0.1[d]	20.5 ± 0.2[b]	20.0 ± 0.1[b]
Day 19	19.6 ± 0.1	19.6 ± 0.2	19.1 ± 0.2*[b]	19.6 ± 0.2	18.1 ± 0.3**	15.4 ± 0.2**
Week 14	18.1 ± 0.1	18.1 ± 0.1	18.5 ± 0.1*	18.5 ± 0.1*	18.9 ± 0.1**	18.1 ± 0.5**
Mean cell hemoglobin concentration (g/dL)						
Day 5	34.7 ± 0.2[b]	34.6 ± 0.1[c]	34.7 ± 0.1	34.5 ± 0.2[d]	34.7 ± 0.2[b]	34.9 ± 0.2[b]
Day 19	34.3 ± 0.2	34.1 ± 0.2	34.0 ± 0.2[b]	34.4 ± 0.3	33.0 ± 0.2**	33.1 ± 0.3**[b]
Week 14	34.4 ± 0.2	34.4 ± 0.2	35.1 ± 0.2	34.8 ± 0.1	34.5 ± 0.1	34.4 ± 0.3
Platelets ($10^3/\mu L$)						
Day 5	999.9 ± 23.9[b]	1,028.6 ± 43.3[c]	1,140.8 ± 31.1*	966.3 ± 51.6[d]	1,008.7 ± 23.2[b]	1,166.4 ± 42.0*[b]
Day 19	763.6 ± 17.9	753.9 ± 24.5	827.4 ± 23.7[b]	777.3 ± 45.5	759.1 ± 30.6	1,815.2 ± 129.0**[b]
Week 14	577.7 ± 21.4	608.0 ± 13.0	597.9 ± 15.1	588.3 ± 18.3	606.1 ± 16.3	673.4 ± 56.4
Leukocytes ($10^3/\mu L$)						
Day 5	7.39 ± 0.79[b]	5.78 ± 0.55[c]	7.81 ± 0.75	6.34 ± 0.43[d]	7.79 ± 0.62[b]	8.33 ± 0.68[b]
Day 19	8.11 ± 0.26	7.22 ± 0.58	8.47 ± 0.41[b]	8.40 ± 0.60	7.66 ± 0.27	9.03 ± 0.49[b]
Week 14	9.68 ± 0.78	9.55 ± 0.54	9.67 ± 0.59	8.88 ± 0.73	8.91 ± 0.64	9.44 ± 0.57
Segmented neutrophils ($10^3/\mu L$)						
Day 5	0.93 ± 0.11	0.73 ± 0.15	0.90 ± 0.15	0.71 ± 0.09	1.14 ± 0.22	1.09 ± 0.10
Day 19	0.85 ± 0.11	0.80 ± 0.10	1.07 ± 0.19	1.09 ± 0.22	0.85 ± 0.08	1.20 ± 0.16
Week 14	1.62 ± 0.16	1.57 ± 0.22	1.45 ± 0.14	1.11 ± 0.20	1.38 ± 0.22	1.01 ± 0.13*

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Male (continued)						
Hematology (continued)						
n						
Day 5	9	6	10	7	8	9
Day 19	10	10	9	10	10	9
Week 14	10	10	10	9	10	10
Immature neutrophils (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.014 ± 0.014	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.011 ± 0.011
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Metamyelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Myelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Promyelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Lymphocytes (10^3/µL)						
Day 5	6.80 ± 0.68	4.77 ± 0.57	6.88 ± 0.63	5.83 ± 0.37	6.89 ± 0.54	7.56 ± 0.54
Day 19	6.99 ± 0.22	6.24 ± 0.53	7.17 ± 0.40	7.14 ± 0.52	6.63 ± 0.23	7.76 ± 0.47
Week 14	8.02 ± 0.66	7.96 ± 0.47	8.16 ± 0.53	7.73 ± 0.61	7.43 ± 0.47	8.41 ± 0.50
Monocytes (10^3/µL)						
Day 5	0.08 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.08 ± 0.03	0.07 ± 0.02
Day 19	0.21 ± 0.03	0.15 ± 0.03	0.20 ± 0.03	0.13 ± 0.03	0.20 ± 0.05	0.18 ± 0.04
Week 14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Basophils (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)						
Day 5	0.01 ± 0.01	0.03 ± 0.03	0.01 ± 0.01	0.03 ± 0.02	0.05 ± 0.04	0.01 ± 0.01
Day 19	0.10 ± 0.04	0.06 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.02 ± 0.01	0.01 ± 0.01*
Week 14	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.09 ± 0.04	0.02 ± 0.01
Methemoglobin (g/dL)						
Day 5	0.03 ± 0.02	0.04 ± 0.02	4.36 ± 0.78**[c]	0.08 ± 0.03**	1.37 ± 0.41**[e]	3.97 ± 0.75**
Day 19	0.09 ± 0.05	0.06 ± 0.02	0.28 ± 0.12*	0.38 ± 0.12**	1.25 ± 0.37**	3.26 ± 0.36**
Week 14	0.03 ± 0.01	0.08 ± 0.01**	0.12 ± 0.02**	0.25 ± 0.07**	0.71 ± 0.20**	3.38 ± 0.80**
Glutathione, erythrocyte (mg/dL erythrocytes)						
Day 5	89.6 ± 5.5	89.7 ± 3.7[e]	98.3 ± 5.0[c]	86.9 ± 6.6	98.3 ± 5.4	101.7 ± 9.6[b]
Day 19	90.3 ± 4.2[c]	95.1 ± 6.4[d]	98.1 ± 6.5[b]	94.1 ± 4.3	104.5 ± 4.9	116.0 ± 8.6*[c]
Week 14	63.7 ± 1.9[e]	67.5 ± 3.6[d]	72.5 ± 3.4[d]	70.7 ± 2.3	72.1 ± 2.5*[c]	73.8 ± 1.9**
Heinz bodies (10^6/µL)[f]						
Day 5	0.002 ± 0.001	0.005 ± 0.001	0.009 ± 0.002*	0.010 ± 0.004	0.007 ± 0.002	0.013 ± 0.004**[d]
Day 19	0.004 ± 0.002	0.004 ± 0.002	0.003 ± 0.001	0.004 ± 0.002	0.006 ± 0.002	0.006 ± 0.002
Week 14	0.170 ± 0.114	0.170 ± 0.114	0.470 ± 0.128	0.267 ± 0.133	0.240 ± 0.122	0.350 ± 0.144

Table F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Male (continued)						
Clinical Chemistry						
n						
Day 5	10	10	10	9	10	10
Day 19	10	10	10	9	10	10
Week 14	10	10	10	10	10	10
Urea nitrogen (mg/dL)						
Day 5	20.4 ± 0.5	18.3 ± 0.7	21.7 ± 0.5	19.7 ± 1.0	19.9 ± 0.6	21.3 ± 0.6
Day 19	21.0 ± 0.7	21.4 ± 0.4	20.8 ± 0.5	22.3 ± 0.5	22.0 ± 0.3	24.9 ± 0.7**
Week 14	17.8 ± 0.5	20.5 ± 0.9*	21.2 ± 0.7**	20.4 ± 0.8*	22.3 ± 1.4**	22.0 ± 0.8**
Creatinine (mg/dL)						
Day 5	0.62 ± 0.01	0.66 ± 0.02	0.72 ± 0.03*	0.62 ± 0.03	0.71 ± 0.04	0.69 ± 0.02
Day 19	0.66 ± 0.03	0.63 ± 0.02	0.64 ± 0.03	0.70 ± 0.03	0.69 ± 0.03	0.76 ± 0.03
Week 14	0.68 ± 0.02	0.70 ± 0.02[c]	0.69 ± 0.02	0.71 ± 0.01	0.69 ± 0.01	0.69 ± 0.02
Total protein (g/dL)						
Day 5	6.5 ± 0.1	6.4 ± 0.1	6.0 ± 0.1**	6.3 ± 0.1**	6.2 ± 0.1*	6.1 ± 0.1**
Day 19	6.7 ± 0.2	7.0 ± 0.1	6.9 ± 0.1	6.7 ± 0.1	6.6 ± 0.1	6.4 ± 0.1
Week 14	6.7 ± 0.1	7.0 ± 0.1	7.0 ± 0.1	7.0 ± 0.1	7.0 ± 0.1	6.5 ± 0.1
Albumin (g/dL)						
Day 5	4.3 ± 0.1	4.3 ± 0.0	4.1 ± 0.0	4.2 ± 0.0	4.2 ± 0.0	4.2 ± 0.0
Day 19	4.4 ± 0.1	4.5 ± 0.0	4.5 ± 0.0	4.4 ± 0.1	4.4 ± 0.1	4.3 ± 0.0
Week 14	4.4 ± 0.0	4.5 ± 0.1	4.6 ± 0.1	4.5 ± 0.1	4.6 ± 0.1	4.4 ± 0.1
Alanine aminotransferase (IU/L)						
Day 5	45 ± 2	44 ± 2	40 ± 2	42 ± 2	45 ± 3	38 ± 2
Day 19	48 ± 3	42 ± 2	46 ± 3	55 ± 6	46 ± 1	45 ± 3
Week 14	52 ± 3	50 ± 3	57 ± 7	51 ± 3	56 ± 6	46 ± 2
Alkaline phosphatase (IU/L)						
Day 5	570 ± 12	586 ± 14	445 ± 8**	544 ± 11*	504 ± 8**	441 ± 5**
Day 19	448 ± 8	455 ± 8	450 ± 8	423 ± 13[b]	402 ± 6**	333 ± 11**
Week 14	216 ± 6	233 ± 9	231 ± 9	209 ± 6	213 ± 6	210 ± 7
Creatine kinase (IU/L)						
Day 5	302 ± 45[c]	397 ± 50	192 ± 32	245 ± 33	295 ± 34	199 ± 18
Day 19	295 ± 80[c]	274 ± 49	221 ± 38[c]	279 ± 79	234 ± 34	337 ± 60
Week 14	120 ± 32	132 ± 40	135 ± 28	184 ± 86	143 ± 27	131 ± 19
Sorbitol dehyrogenase (IU/L)						
Day 5	9 ± 1	13 ± 1	12 ± 2	11 ± 2	12 ± 3	12 ± 1
Day 19	12 ± 1	11 ± 1	11 ± 0	13 ± 2[b]	12 ± 1	14 ± 1
Week 14	14 ± 3	11 ± 4	17 ± 2	17 ± 4	19 ± 4	19 ± 4
Bile acids (µmol/L)						
Day 5	13.8 ± 2.9	24.6 ± 5.0*	12.9 ± 1.7	16.3 ± 3.9	14.0 ± 2.7	8.8 ± 1.3
Day 19	15.3 ± 4.6	14.2 ± 4.7	13.6 ± 2.1[c]	13.4 ± 2.3	7.9 ± 0.6	12.5 ± 1.9
Week 14	5.7 ± 0.7[c]	6.3 ± 0.5[g]	13.6 ± 4.7[d]	13.9 ± 1.8**	9.7 ± 2.6[e]	5.8 ± 0.5[g]

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Female						
Hematology						
n						
Day 5	9	8	9	9	8	9
Day 19	10	10	10	10	10	10
Week 14	10	10	10	10	9	10
Hematocrit (%)						
Day 5	45.6 ± 1.4	44.3 ± 1.2	46.0 ± 0.9[b]	46.0 ± 0.7	45.1 ± 1.0[c]	44.8 ± 0.6
Day 19	46.8 ± 0.5	46.0 ± 0.6	45.2 ± 0.5	46.6 ± 0.8	48.5 ± 1.3	42.7 ± 1.8
Week 14	43.8 ± 0.4	43.5 ± 0.4	42.1 ± 0.4	43.0 ± 0.4	47.1 ± 0.4**	51.3 ± 0.6**
Hemoglobin (g/dL)						
Day 5	15.3 ± 0.4	14.8 ± 0.3	15.4 ± 0.2[b]	15.4 ± 0.2	15.3 ± 0.2[c]	15.2 ± 0.1
Day 19	15.7 ± 0.1	15.3 ± 0.1	15.2 ± 0.2	15.6 ± 0.2	15.9 ± 0.4	13.9 ± 0.6*
Week 14	15.2 ± 0.1	15.0 ± 0.1	14.7 ± 0.1	15.0 ± 0.1	16.2 ± 0.1**	17.5 ± 0.1**
Erythrocytes (10^6/µL)						
Day 5	7.62 ± 0.26	7.27 ± 0.21	7.64 ± 0.13[b]	7.56 ± 0.13	7.44 ± 0.20[c]	7.49 ± 0.09
Day 19	7.87 ± 0.12	7.72 ± 0.13	7.54 ± 0.08	7.83 ± 0.17	8.36 ± 0.11*	8.71 ± 0.20**
Week 14	7.64 ± 0.07	7.61 ± 0.05	7.31 ± 0.06	7.35 ± 0.07	7.74 ± 0.05	8.49 ± 0.15**
Reticulocytes (10^6/µL)						
Day 5	0.29 ± 0.03	0.34 ± 0.02	0.36 ± 0.03	0.44 ± 0.05*	0.63 ± 0.03**	0.73 ± 0.07**
Day 19	0.14 ± 0.01	0.17 ± 0.01*	0.14 ± 0.01	0.20 ± 0.02**	0.41 ± 0.05**	0.65 ± 0.07**
Week 14	0.21 ± 0.02	0.15 ± 0.02	0.18 ± 0.02	0.23 ± 0.03	0.28 ± 0.03	0.40 ± 0.03**
Nucleated erythrocytes (10^3/µL)						
Day 5	1.80 ± 0.44[b]	1.13 ± 0.35	1.44 ± 0.48	1.56 ± 0.53	2.40 ± 0.43[b]	2.22 ± 0.36
Day 19	0.60 ± 0.22	0.40 ± 0.16	0.90 ± 0.48	1.10 ± 0.38	0.60 ± 0.27	3.00 ± 0.78*
Week 14	1.00 ± 0.30	0.60 ± 0.22	0.80 ± 0.25	1.60 ± 0.34	1.00 ± 0.37	1.70 ± 0.42
Mean cell volume (fL)						
Day 5	59.9 ± 0.4	60.9 ± 0.3	60.3 ± 0.4[b]	60.8 ± 0.3	60.7 ± 0.5[c]	59.8 ± 0.3
Day 19	59.5 ± 0.5	59.6 ± 0.6	60.0 ± 0.4	59.5 ± 0.5	58.0 ± 1.0	48.9 ± 1.0**
Week 14	57.2 ± 0.1	57.2 ± 0.3	57.6 ± 0.2	58.6 ± 0.2**	60.8 ± 0.2**	60.5 ± 0.7**
Mean cell hemoglobin (pg)						
Day 5	20.1 ± 0.2	20.3 ± 0.2	20.1 ± 0.2[b]	20.3 ± 0.1	20.7 ± 0.3[c]	20.3 ± 0.1
Day 19	19.9 ± 0.2	19.9 ± 0.2	20.1 ± 0.1	19.9 ± 0.3	19.0 ± 0.4	15.8 ± 0.3**
Week 14	19.9 ± 0.1	19.7 ± 0.1	20.1 ± 0.1	20.5 ± 0.1**	20.9 ± 0.1**	20.7 ± 0.3**
Mean cell hemoglobin concentration (g/dL)						
Day 5	33.6 ± 0.3	33.3 ± 0.2	33.4 ± 0.3[b]	33.4 ± 0.2	34.0 ± 0.3[c]	33.9 ± 0.3
Day 19	33.5 ± 0.2	33.4 ± 0.2	33.6 ± 0.1	33.4 ± 0.3	32.9 ± 0.2*	32.4 ± 0.2**
Week 14	34.7 ± 0.2	34.4 ± 0.1	34.9 ± 0.2	34.9 ± 0.2	34.3 ± 0.2	34.2 ± 0.2
Platelets (10^3/µL)						
Day 5	794.6 ± 41.0	891.1 ± 27.6	847.3 ± 35.4[b]	866.9 ± 16.3	995.3 ± 34.5**[c]	931.8 ± 43.9**
Day 19	701.8 ± 24.7	703.4 ± 22.4	698.4 ± 30.7	709.4 ± 16.3	726.7 ± 61.2	1,631.0 ± 171.7**
Week 14	656.6 ± 17.0	611.5 ± 10.8	668.7 ± 21.3	613.6 ± 11.3	645.3 ± 21.9	617.5 ± 14.9
Leukocytes (10^3/µL)						
Day 5	9.16 ± 0.76	9.41 ± 1.06	10.03 ± 0.68[b]	8.16 ± 0.98	9.19 ± 0.60[c]	9.34 ± 0.93
Day 19	10.55 ± 0.54	9.55 ± 0.67	9.28 ± 0.48	9.18 ± 0.44	8.86 ± 0.56	10.72 ± 0.63
Week 14	9.61 ± 0.20	10.03 ± 0.46	8.60 ± 0.50	8.63 ± 0.48	11.29 ± 0.63	9.72 ± 0.41
Segmented neutrophils (10^3/µL)						
Day 5	0.98 ± 0.16	1.03 ± 0.15	0.90 ± 0.15	1.06 ± 0.14	1.04 ± 0.19	0.97 ± 0.17
Day 19	0.85 ± 0.10	0.75 ± 0.11	1.13 ± 0.14	0.95 ± 0.08	0.73 ± 0.11	0.93 ± 0.11
Week 14	1.53 ± 0.21	1.56 ± 0.22	1.61 ± 0.16	1.54 ± 0.11	1.86 ± 0.28	1.42 ± 0.10

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Female (continued)						
Hematology (continued)						
n						
Day 5	9	8	9	9	8	9
Day 19	10	10	10	10	10	10
Week 14	10	10	10	10	9	10
Immature neutrophils (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.011 ± 0.011	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.010 ± 0.010	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Metamyelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Myelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Promyelocytes (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Lymphocytes (10^3/µL)						
Day 5	8.11 ± 0.68	8.23 ± 0.94	9.00 ± 0.73	7.00 ± 0.90	8.18 ± 0.53	8.28 ± 0.78
Day 19	9.33 ± 0.46	8.52 ± 0.62	7.82 ± 0.48	7.98 ± 0.42	7.86 ± 0.51	9.59 ± 0.63
Week 14	7.93 ± 0.21	8.32 ± 0.52	6.93 ± 0.41	6.92 ± 0.38	9.30 ± 0.50	8.16 ± 0.48
Monocytes (10^3/µL)						
Day 5	0.03 ± 0.02	0.01 ± 0.01	0.02 ± 0.02	0.04 ± 0.02	0.10 ± 0.02	0.07 ± 0.04
Day 19	0.29 ± 0.06	0.22 ± 0.07	0.29 ± 0.06	0.22 ± 0.05	0.22 ± 0.04	0.18 ± 0.04
Week 14	0.09 ± 0.03	0.03 ± 0.02	0.02 ± 0.01	0.06 ± 0.04	0.02 ± 0.02	0.08 ± 0.03
Basophils (10^3/µL)						
Day 5	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Day 19	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Week 14	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Eosinophils (10^3/µL)						
Day 5	0.04 ± 0.02	0.14 ± 0.04	0.06 ± 0.02	0.04 ± 0.02	0.03 ± 0.02	0.06 ± 0.03
Day 19	0.06 ± 0.02	0.05 ± 0.02	0.08 ± 0.04	0.05 ± 0.02	0.06 ± 0.02	0.01 ± 0.01
Week 14	0.08 ± 0.03	0.13 ± 0.04	0.07 ± 0.03	0.11 ± 0.03	0.11 ± 0.04	0.09 ± 0.04
Methemoglobin (g/dL)						
Day 5	0.02 ± 0.01	0.10 ± 0.05*	0.05 ± 0.01	0.21 ± 0.08**	2.41 ± 0.76**	4.95 ± 0.92**
Day 19	0.03 ± 0.01	0.11 ± 0.03*	0.18 ± 0.04**	2.01 ± 0.49**	3.78 ± 0.79**	6.66 ± 0.36**
Week 14	0.06 ± 0.02	0.14 ± 0.02**	0.16 ± 0.02**	0.48 ± 0.05**[c]	0.99 ± 0.20**	2.27 ± 0.54**
Glutathione, erythrocyte (mg/dL erythrocytes)						
Day 5	83.4 ± 6.6[d]	86.8 ± 6.7[g]	81.5 ± 6.2[d]	87.6 ± 4.3[d]	91.1 ± 4.9[c]	105.0 ± 5.5[d]
Day 19	88.3 ± 5.4	83.0 ± 4.8[d]	79.6 ± 5.5[c]	89.8 ± 4.2[d]	84.0 ± 1.7[e]	98.3 ± 3.8
Week 14	77.9 ± 4.2[d]	79.2 ± 2.1[c]	85.4 ± 2.4[d]	88.3 ± 1.6	84.2 ± 3.4	77.3 ± 2.6[d]
Heinz bodies (10^6/µL)[f]						
Day 5	0.002 ± 0.001	0.001 ± 0.001	0.002 ± 0.001	0.002 ± 0.001	0.006 ± 0.002	0.010 ± 0.002**
Day 19	0.002 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.003 ± 0.002	0.002 ± 0.002	0.002 ± 0.001
Week 14	0.300 ± 0.123	0.230 ± 0.117	0.350 ± 0.117	0.210 ± 0.107	0.178 ± 0.118	0.240 ± 0.122

TABLE F1
Hematology and Clinical Chemistry Data for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Female (continued)						
Clinical Chemistry						
n						
Day 5	10	9	9	9	10	10
Day 19	10	10	10	10	10	10
Week 14	10	10	10	10	9	10
Urea nitrogen (mg/dL)						
Day 5	22.1 ± 1.1	21.2 ± 0.6	21.3 ± 0.6	20.3 ± 0.7[d]	21.0 ± 0.5	22.5 ± 0.6
Day 19	24.4 ± 1.0[c]	24.2 ± 0.7	23.2 ± 0.5	23.9 ± 0.5	24.1 ± 0.6	30.6 ± 0.8**
Week 14	17.8 ± 0.6	19.0 ± 0.4	19.0 ± 0.7	21.7 ± 0.4**	22.3 ± 0.7**	21.5 ± 0.5**
Creatinine (mg/dL)						
Day 5	0.63 ± 0.03[c]	0.62 ± 0.03	0.70 ± 0.04	0.65 ± 0.02[d]	0.71 ± 0.01*[c]	0.73 ± 0.03*[c]
Day 19	0.58 ± 0.04[d]	0.65 ± 0.03*	0.60 ± 0.02	0.69 ± 0.03**	0.73 ± 0.04**	0.81 ± 0.03**
Week 14	0.66 ± 0.02	0.68 ± 0.03	0.67 ± 0.02	0.68 ± 0.03	0.68 ± 0.03	0.69 ± 0.02
Total protein (g/dL)						
Day 5	6.1 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.3 ± 0.1	6.2 ± 0.1	6.1 ± 0.1
Day 19	6.1 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.3 ± 0.2	6.1 ± 0.1	6.2 ± 0.1
Week 14	6.8 ± 0.1	7.2 ± 0.1	7.0 ± 0.1	7.1 ± 0.1	7.0 ± 0.1	6.7 ± 0.1
Albumin (g/dL)						
Day 5	4.2 ± 0.1	4.4 ± 0.1	4.4 ± 0.1	4.5 ± 0.1*[d]	4.5 ± 0.1	4.4 ± 0.1
Day 19	4.3 ± 0.1	4.5 ± 0.0	4.5 ± 0.0	4.5 ± 0.1	4.4 ± 0.1	4.5 ± 0.1
Week 14	4.5 ± 0.0	4.8 ± 0.1	4.8 ± 0.1	4.8 ± 0.1*	4.7 ± 0.1	4.8 ± 0.1
Alanine aminotransferase (IU/L)						
Day 5	41 ± 4	37 ± 1	40 ± 2[b]	38 ± 2	37 ± 1	44 ± 3
Day 19	38 ± 2	35 ± 1	33 ± 2	37 ± 2	38 ± 2	40 ± 1
Week 14	45 ± 2	42 ± 2	43 ± 2	53 ± 3	53 ± 2*	48 ± 3
Alkaline phosphatase (IU/L)						
Day 5	424 ± 21	423 ± 15	415 ± 14[b]	377 ± 13	345 ± 11**	315 ± 12**
Day 19	350 ± 10	299 ± 22*	340 ± 5	315 ± 16*	288 ± 16**	257 ± 4**
Week 14	178 ± 5	176 ± 8	180 ± 5	167 ± 3	164 ± 3	142 ± 5**
Creatine kinase (IU/L)						
Day 5	344 ± 60[c]	396 ± 94	398 ± 71[d]	397 ± 62	321 ± 54[c]	468 ± 75[d]
Day 19	288 ± 67[e]	267 ± 28	292 ± 40	229 ± 21	383 ± 52	391 ± 42[c]
Week 14	99 ± 22	71 ± 14	67 ± 10	69 ± 14	60 ± 9	109 ± 11
Sorbitol dehydrogenase (IU/L)						
Day 5	11 ± 1	9 ± 1	11 ± 0[b]	10 ± 1	10 ± 1	13 ± 2
Day 19	13 ± 0	13 ± 0	12 ± 1	13 ± 1	14 ± 1	14 ± 1
Week 14	12 ± 5	7 ± 1	6 ± 1	8 ± 1	7 ± 1	10 ± 1
Bile acids (µmol/L)						
Day 5	12.7 ± 2.5[c]	12.4 ± 2.1	21.0 ± 5.9[d]	16.3 ± 5.0[d]	13.6 ± 2.2[c]	17.4 ± 3.5[d]
Day 19	21.1 ± 5.8[c]	15.4 ± 2.3	16.8 ± 3.0	12.9 ± 1.4	14.8 ± 2.5	15.8 ± 3.0
Week 14	17.6 ± 2.2	20.2 ± 3.6	25.1 ± 4.2	30.0 ± 8.6	25.0 ± 3.4	32.0 ± 7.7

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

** $P \leq 0.01$

[a] Mean ± standard error. Statistical tests were performed on unrounded data.

[b] n=10

[c] n=9

[d] n=8

[e] n=7

[f] Total number of erythrocytes with Heinz bodies

[g] n=6

TABLE F2
Hemoglobin, Methemoglobin, and Serum and Gastric Nitrosamine Data for Rats on Days 70 and 71 in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
n	5	5	5	5	5	5
Male						
Hemoglobin (g/dL)						
Day 70 (at 2000 hours)	14.0 ± 0.8	14.4 ± 0.7[b]	14.9 ± 0.2	15.0 ± 0.2	16.1 ± 0.4	14.6 ± 0.8
Day 70 (at 2200 hours)	15.2 ± 0.2	15.4 ± 0.3	15.1 ± 0.2	15.3 ± 0.4[c]	16.7 ± 0.6	15.2 ± 0.5
Day 71 (at 0900 hours)	15.4 ± 0.2	15.4 ± 0.2	15.3 ± 0.1	15.0 ± 0.2	15.9 ± 0.2[b]	14.4 ± 0.1*
Methemoglobin (g/dL)						
Day 70 (at 2000 hours)	0.05 ± 0.02	0.09 ± 0.02[b]	0.12 ± 0.02*	0.44 ± 0.17**	0.47 ± 0.27**	0.54 ± 0.09**
Day 70 (at 2200 hours)	0.02 ± 0.01	0.05 ± 0.01	0.07 ± 0.01**	0.14 ± 0.00**[c]	0.70 ± 0.48**	0.64 ± 0.23**
Day 71 (at 0900 hours)	0.03 ± 0.01	0.09 ± 0.01**	0.10 ± 0.03*	0.54 ± 0.23**	3.50 ± 0.68**[b]	5.13 ± 1.40**
Serum nitrosamine						
Day 70 (at 2000 hours)	—[d]	—	—	—	—	—
Day 70 (at 2200 hours)	—	—	—	—	—	—
Day 71 (at 0900 hours)	—	—	—	—	—	—
Gastric nitrosamine						
Day 70 (at 2000 hours)	—	—	—	—	—	—
Day 70 (at 2200 hours)	—	—	—	—	—	—
Day 71 (at 0900 hours)	—	—	—	—	—	—
Female						
Hemoglobin (g/dL)						
Day 70 (at 2000 hours)	16.2 ± 0.3	15.4 ± 0.2	15.1 ± 0.1	15.8 ± 0.4	17.5 ± 0.2	15.5 ± 0.5[b]
Day 70 (at 2200 hours)	15.7 ± 0.4	15.5 ± 0.3[b]	15.4 ± 0.2	15.8 ± 0.3	16.8 ± 0.2*	16.7 ± 0.0[c]
Day 71 (at 0900 hours)	15.7 ± 0.2	15.2 ± 0.2	15.8 ± 0.1	15.2 ± 0.2	16.5 ± 0.1*	17.2 ± 0.8[b]
Methemoglobin (g/dL)						
Day 70 (at 2000 hours)	0.04 ± 0.02	0.21 ± 0.08*	0.39 ± 0.12**	0.60 ± 0.11**	0.57 ± 0.20**	2.76 ± 0.75**[b]
Day 70 (at 2200 hours)	0.27 ± 0.09	0.12 ± 0.02[b]	0.26 ± 0.06[b]	0.26 ± 0.05	0.50 ± 0.10	0.44 ± 0.07[c]
Day 71 (at 0900 hours)	0.09 ± 0.02	0.19 ± 0.05*	1.16 ± 0.30**	1.06 ± 0.09**	4.39 ± 0.79**	3.32 ± 1.78**[b]
Serum nitrosamine						
Day 70 (at 2000 hours)	—	—	—	—	—	—
Day 70 (at 2200 hours)	—	—	—	—	—	—
Day 71 (at 0900 hours)	—	—	—	—	—	—
Gastric nitrosamine						
Day 70 (at 2000 hours)	—	—	—	—	—	—
Day 70 (at 2200 hours)	—	—	—	—	—	—
Day 71 (at 0900 hours)	—	—	—	—	—	—

* Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test

** $P \leq 0.01$

[a] Mean ± standard error. Statistical tests were performed on unrounded data.

[b] n=4

[c] n=3

[d] Undetectable by thermal energy analyzer. Nitrosamine includes *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodipropylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine, *N*-nitrosopyrrolidine, and *N*-nitrosomorpholine concentrations.

APPENDIX G
ORGAN WEIGHTS AND ORGAN-WEIGHT-TO-BODY-WEIGHT RATIOS

TABLE G1 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Drinking Water Study of Sodium Nitrite** 230
TABLE G2 **Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 14-Week Drinking Water Study of Sodium Nitrite** 232

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Male						
n	10	10	10	10	10	10
Necropsy body wt	372 ± 5	357 ± 7	360 ± 9	366 ± 4	347 ± 6*	316 ± 8**
Heart						
Absolute	1.109 ± 0.014	1.056 ± 0.023	1.106 ± 0.034	1.123 ± 0.019	1.113 ± 0.021	1.078 ± 0.039
Relative	0.298 ± 0.004	0.296 ± 0.005	0.307 ± 0.006	0.307 ± 0.005	0.321 ± 0.004**	0.340 ± 0.006**
R. Kidney						
Absolute	1.273 ± 0.016	1.293 ± 0.036	1.276 ± 0.033	1.319 ± 0.021	1.328 ± 0.030	1.250 ± 0.029
Relative	0.342 ± 0.003	0.362 ± 0.007*	0.354 ± 0.003*	0.360 ± 0.004*	0.382 ± 0.005**	0.396 ± 0.006**
Liver						
Absolute	13.140 ± 0.412[b]	13.252 ± 0.394	13.098 ± 0.351	13.222 ± 0.187	12.593 ± 0.367	11.086 ± 0.343**
Relative	3.522 ± 0.066[b]	3.712 ± 0.085	3.638 ± 0.051	3.610 ± 0.048	3.622 ± 0.066	3.509 ± 0.075
Lung						
Absolute	1.796 ± 0.051	1.785 ± 0.075	1.675 ± 0.069	1.756 ± 0.082	1.746 ± 0.057	1.575 ± 0.056
Relative	0.483 ± 0.016	0.499 ± 0.016	0.465 ± 0.014	0.479 ± 0.022	0.502 ± 0.010	0.497 ± 0.008
Spleen						
Absolute	0.822 ± 0.006	0.793 ± 0.019	0.829 ± 0.018	0.849 ± 0.015	0.881 ± 0.019	0.818 ± 0.025
Relative	0.221 ± 0.003	0.222 ± 0.003	0.230 ± 0.003	0.232 ± 0.003*	0.254 ± 0.004**	0.259 ± 0.004**
R. Testis						
Absolute	1.686 ± 0.127	1.515 ± 0.024	1.539 ± 0.022	1.575 ± 0.016	1.553 ± 0.018	1.497 ± 0.019
Relative	0.452 ± 0.032	0.425 ± 0.005	0.429 ± 0.009	0.430 ± 0.006	0.448 ± 0.009	0.476 ± 0.009
Thymus						
Absolute	0.366 ± 0.018	0.363 ± 0.013	0.367 ± 0.011	0.396 ± 0.010	0.385 ± 0.019	0.328 ± 0.015
Relative	0.098 ± 0.005	0.102 ± 0.003	0.102 ± 0.004	0.108 ± 0.003	0.111 ± 0.006	0.104 ± 0.006

TABLE G1
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Rats in the 14-Week Drinking Water Study of Sodium Nitrite

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
Female						
n	10	10	10	10	9	10
Necropsy body wt	207 ± 4	207 ± 2	200 ± 3	207 ± 3	194 ± 3*	185 ± 6**
Heart						
Absolute	0.711 ± 0.018	0.716 ± 0.013	0.700 ± 0.020	0.734 ± 0.015	0.713 ± 0.009	0.742 ± 0.009
Relative	0.344 ± 0.004	0.345 ± 0.005	0.350 ± 0.006	0.355 ± 0.005	0.369 ± 0.006*	0.406 ± 0.014**
R. Kidney						
Absolute	0.778 ± 0.017	0.758 ± 0.017	0.768 ± 0.012	0.793 ± 0.013	0.819 ± 0.022	0.841 ± 0.017**
Relative	0.376 ± 0.003	0.365 ± 0.006	0.385 ± 0.003	0.383 ± 0.005	0.423 ± 0.008**	0.460 ± 0.017**
Liver						
Absolute	6.376 ± 0.178	6.814 ± 0.164	6.730 ± 0.200	6.730 ± 0.119	6.324 ± 0.133	6.155 ± 0.167
Relative	3.086 ± 0.061	3.286 ± 0.073	3.371 ± 0.078	3.256 ± 0.041	3.268 ± 0.063	3.364 ± 0.141
Lung						
Absolute	1.281 ± 0.066	1.214 ± 0.043	1.273 ± 0.054	1.301 ± 0.044	1.284 ± 0.063	1.160 ± 0.024
Relative	0.621 ± 0.032	0.585 ± 0.020	0.636 ± 0.020	0.628 ± 0.014	0.662 ± 0.028	0.633 ± 0.021
Spleen						
Absolute	0.532 ± 0.009	0.523 ± 0.006	0.514 ± 0.007	0.552 ± 0.014	0.612 ± 0.013**	0.654 ± 0.013**
Relative	0.258 ± 0.004	0.252 ± 0.003	0.258 ± 0.003	0.267 ± 0.005	0.317 ± 0.006**	0.357 ± 0.013**
Thymus						
Absolute	0.276 ± 0.011	0.276 ± 0.011	0.272 ± 0.008	0.284 ± 0.006	0.279 ± 0.014	0.270 ± 0.006
Relative	0.134 ± 0.006	0.133 ± 0.005	0.137 ± 0.004	0.138 ± 0.002	0.144 ± 0.007	0.148 ± 0.006

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* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as g organ weight/g body weight as a percentage (mean ± standard error).

[b] n=9

TABLE G2
Organ Weights and Organ-Weight-to-Body-Weight Ratios for Mice in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	750 ppm	1,500 ppm	3,000 ppm	5,000 ppm
n	10	10	10	10	10	10
Male						
Necropsy body wt	34.6 ± 0.8	34.4 ± 0.8	33.5 ± 0.7	33.4 ± 1.0	31.7 ± 1.0*	30.7 ± 0.8**
Heart						
Absolute	0.166 ± 0.006	0.173 ± 0.005	0.168 ± 0.007	0.166 ± 0.007	0.162 ± 0.003	0.169 ± 0.008
Relative	0.480 ± 0.019	0.502 ± 0.012	0.502 ± 0.014	0.499 ± 0.017	0.515 ± 0.018	0.552 ± 0.030*
R. Kidney						
Absolute	0.296 ± 0.007	0.291 ± 0.004	0.284 ± 0.008	0.285 ± 0.008	0.288 ± 0.007	0.291 ± 0.008
Relative	0.859 ± 0.023	0.851 ± 0.024	0.850 ± 0.017	0.856 ± 0.019	0.912 ± 0.026	0.952 ± 0.018**
Liver						
Absolute	1.630 ± 0.042	1.530 ± 0.142	1.652 ± 0.041	1.450 ± 0.046	1.648 ± 0.058	1.616 ± 0.075
Relative	4.718 ± 0.107	4.400 ± 0.388	4.944 ± 0.114	4.349 ± 0.079	5.200 ± 0.108	5.257 ± 0.168
Lung						
Absolute	0.295 ± 0.013	0.267 ± 0.020	0.273 ± 0.013	0.277 ± 0.014	0.269 ± 0.011	0.276 ± 0.017
Relative	0.852 ± 0.031	0.772 ± 0.049	0.815 ± 0.030	0.836 ± 0.047	0.860 ± 0.052	0.898 ± 0.043
Spleen						
Absolute	0.071 ± 0.003	0.071 ± 0.002	0.072 ± 0.002	0.074 ± 0.002	0.080 ± 0.003	0.106 ± 0.005**
Relative	0.206 ± 0.007	0.209 ± 0.007	0.217 ± 0.006	0.221 ± 0.007	0.253 ± 0.009**	0.344 ± 0.013**
R. Testis						
Absolute	0.124 ± 0.003	0.123 ± 0.002	0.117 ± 0.002	0.121 ± 0.002	0.117 ± 0.003	0.122 ± 0.002
Relative	0.358 ± 0.010	0.358 ± 0.007	0.350 ± 0.004	0.364 ± 0.010	0.372 ± 0.011	0.398 ± 0.008**
Thymus						
Absolute	0.062 ± 0.003	0.060 ± 0.004	0.051 ± 0.005	0.050 ± 0.003	0.050 ± 0.004	0.052 ± 0.003
Relative	0.181 ± 0.010	0.174 ± 0.012	0.152 ± 0.015	0.150 ± 0.005	0.158 ± 0.014	0.170 ± 0.009
Female						
Necropsy body wt	30.1 ± 0.9	31.4 ± 1.0	31.8 ± 1.2	31.3 ± 1.0	31.7 ± 1.1	28.2 ± 0.6
Heart						
Absolute	0.143 ± 0.005	0.139 ± 0.006	0.159 ± 0.006	0.156 ± 0.005	0.164 ± 0.006*	0.168 ± 0.006**
Relative	0.477 ± 0.021	0.443 ± 0.017	0.502 ± 0.022	0.501 ± 0.020	0.521 ± 0.020	0.598 ± 0.021**
R. Kidney						
Absolute	0.198 ± 0.004	0.196 ± 0.005	0.208 ± 0.005	0.201 ± 0.004	0.214 ± 0.005*	0.212 ± 0.004*
Relative	0.659 ± 0.014	0.627 ± 0.012	0.657 ± 0.014	0.644 ± 0.013	0.677 ± 0.016	0.753 ± 0.006**
Liver						
Absolute	1.454 ± 0.030	1.504 ± 0.031	1.617 ± 0.053	1.487 ± 0.035	1.682 ± 0.041**	1.575 ± 0.042**
Relative	4.853 ± 0.112	4.822 ± 0.120	5.096 ± 0.091	4.785 ± 0.143	5.328 ± 0.096**	5.597 ± 0.137**
Lung						
Absolute	0.296 ± 0.009	0.293 ± 0.017	0.311 ± 0.013	0.289 ± 0.014	0.274 ± 0.018	0.254 ± 0.019
Relative	0.989 ± 0.037	0.938 ± 0.050	0.986 ± 0.049	0.934 ± 0.053	0.871 ± 0.061	0.899 ± 0.061
Spleen						
Absolute	0.090 ± 0.003	0.090 ± 0.003	0.098 ± 0.002	0.096 ± 0.003	0.125 ± 0.004**	0.149 ± 0.008**
Relative	0.300 ± 0.010	0.290 ± 0.012	0.312 ± 0.012	0.311 ± 0.015	0.396 ± 0.014**	0.531 ± 0.028**
Thymus						
Absolute	0.061 ± 0.004	0.064 ± 0.004	0.065 ± 0.004	0.066 ± 0.003	0.065 ± 0.003	0.056 ± 0.003
Relative	0.204 ± 0.012	0.204 ± 0.009	0.206 ± 0.013	0.211 ± 0.006	0.208 ± 0.012	0.198 ± 0.011

* Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test

** $P \leq 0.01$

[a] Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as g organ weight/g body weight as a percentage (mean ± standard error).

APPENDIX H
REPRODUCTIVE TISSUE EVALUATIONS AND ESTROUS CYCLE CHARACTERIZATION

TABLE H1 **Summary of Reproductive Tissue Evaluations for Male Rats in the 14-Week Drinking Water Study of Sodium Nitrite** 234

TABLE H2 **Estrous Cycle Characterization for Female Rats in the 14-Week Drinking Water Study of Sodium Nitrite** 234

TABLE H3 **Summary of Reproductive Tissue Evaluations for Male Mice in the 14-Week Drinking Water Study of Sodium Nitrite** 235

TABLE H4 **Estrous Cycle Characterization for Female Mice in the 14-Week Drinking Water Study of Sodium Nitrite** 235

TABLE H1
Summary of Reproductive Tissue Evaluations for Male Rats in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	1,500 ppm	5,000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	372 ± 5	357 ± 7	366 ± 4	316 ± 8**
L. Cauda epididymis	0.2030 ± 0.0066	0.1976 ± 0.0044	0.1860 ± 0.0042	0.1900 ± 0.0050
L. Epididymis	0.5110 ± 0.0133	0.5017 ± 0.0067	0.4922 ± 0.0090	0.4745 ± 0.0072*
L. Testis	1.5359 ± 0.0609	1.4418 ± 0.0719	1.6062 ± 0.0145	1.5386 ± 0.0249
Spermatid measurements				
Spermatid heads (10^7/g testis)	9.471 ± 0.562	10.722 ± 1.046	10.268 ± 0.733	9.309 ± 0.488
Spermatid heads (10^7/testis)	14.310 ± 0.540	14.865 ± 0.645	16.560 ± 1.282	14.325 ± 0.773
Spermatid count (mean/10^{-4} mL suspension)	71.550 ± 2.702	74.325 ± 3.227	82.800 ± 6.412	71.625 ± 3.863
Epididymal spermatozoal measurements				
Motility (%)	89.45 ± 1.85	91.09 ± 1.38	83.18 ± 2.15*	73.42 ± 4.15**
Concentration (10^6/g cauda epididymal tissue)	366 ± 15[b]	370 ± 14	366 ± 14	391 ± 15

* Significantly different ($P \leq 0.05$) from the control group by Williams' test (epididymis weight) or Shirley's test (motility)

** Significantly different ($P \leq 0.01$) from the control group by Williams' test (body weight) or Shirley's test (motility)

[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (cauda epididymis and testis weights) or Dunn's test (spermatid measurements, epididymal spermatozoal concentration).

[b] n=9

TABLE H2
Estrous Cycle Characterization for Female Rats in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	750 ppm	3,000 ppm
n	10	10	10	9
Necropsy body wt (g)	205 ± 4	207 ± 2	200 ± 3	194 ± 3*
Estrous cycle length (days)	5.100 ± 0.125	4.850 ± 0.076	5.050 ± 0.117	5.278 ± 0.121
Estrous stages (% of cycle)				
Diestrus	40.8	40.0	42.5	42.6
Proestrus	15.0	15.0	12.5	15.7
Estrus	23.3	25.0	25.8	21.3
Metestrus	20.8	20.0	18.3	20.4
Uncertain diagnoses	0.0	0.0	0.8	0.0

* Significantly different ($P \leq 0.05$) from the control group by Williams' test

[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for estrous cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages.

TABLE H3
Summary of Reproductive Tissue Evaluations for Male Mice in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	1,500 ppm	5,000 ppm
n	10	10	10	10
Weights (g)				
Necropsy body wt	34.6 ± 0.8	34.4 ± 0.8	33.4 ± 0.9	30.7 ± 0.8**
L. Cauda epididymis	0.0287 ± 0.0035	0.0254 ± 0.0032	0.0273 ± 0.0038	0.0256 ± 0.0032
L. Epididymis	0.0613 ± 0.0060	0.0549 ± 0.0027	0.0587 ± 0.0040	0.0601 ± 0.0074
L. Testis	0.1222 ± 0.0039	0.1216 ± 0.0049	0.1236 ± 0.0034	0.1167 ± 0.0027
Spermatid measurements				
Spermatid heads (10^7/g testis)	18.274 ± 0.635	17.016 ± 0.891	18.566 ± 0.702	18.012 ± 1.017
Spermatid heads (10^7/testis)	2.228 ± 0.092	2.060 ± 0.111	2.287 ± 0.091	2.094 ± 0.112
Spermatid count (mean/10^{-4} mL suspension)	69.625 ± 2.864	64.350 ± 3.485	71.500 ± 2.853	65.425 ± 3.519
Epididymal spermatozoal measurements				
Motility (%)	73.89 ± 4.14	71.74 ± 3.63	67.86 ± 1.57	39.23 ± 7.41**
Concentration (10^6/g cauda epididymal tissue)	527 ± 93	685 ± 74	457 ± 106	717 ± 99

** Significantly different ($P \leq 0.01$) from the control group by Williams' test (body weight) or Shirley's test (motility)

[a] Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (tissue weights) or Dunn's test (spermatid measurements, epididymal spermatozoal concentration).

TABLE H4
Estrous Cycle Characterization for Female Mice in the 14-Week Drinking Water Study of Sodium Nitrite[a]

	0 ppm	375 ppm	1,500 ppm	5,000 ppm
n	10	10	10	10
Necropsy body wt (g)	30.1 ± 0.9	31.4 ± 1.0	31.3 ± 1.0	28.2 ± 0.6
Estrous cycle length (days)	4.188 ± 0.132[b]	4.400 ± 0.125	4.667 ± 0.118*[c]	4.813 ± 0.091**[b]
Estrous stages (% of cycle)				
Diestrus	37.5	29.2	30.8	32.5
Proestrus	20.8	20.8	19.2	18.3
Estrus	25.0	30.8	35.8	33.3
Metestrus	16.7	19.2	14.2	15.8

* Significantly different ($P \leq 0.05$) from the control group by Shirley's test

** $P \leq 0.01$

[a] Necropsy body weight and estrous cycle length data are presented as mean ± standard error. Differences from the control group for necropsy cycle length are not significant by Dunn's test. By multivariate analysis of variance, exposed females do not differ significantly from the control females in the relative length of time spent in the estrous stages.

[b] Estrous cycle was longer than 12 days or unclear in 2 of 10 animals.

[c] Estrous cycle was longer than 12 days or unclear in 1 of 10 animals.

APPENDIX I
DETERMINATIONS OF PLASMA NITRITE AND BLOOD METHEMOGLOBIN CONCENTRATIONS

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TABLE I1
Plasma Nitrite and Blood Methemoglobin Concentrations at 2 Weeks in Male Rats in the 2-Year Drinking Water Study of Sodium Nitrite[a]

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Plasma Nitrite (µg/mL)				
Time of collection				
0600	—[b]	—	—	4.500[c]
1200	—	—	—	—
2100	—	—	2.100[c]	9.000 ± 2.000
2400	—	2.000[c]	4.200 ± 0.300	—
0300	—	—	—	8.100 ± 2.900
Blood Methemoglobin (g/dL)				
Time of collection				
0600	0.250 ± 0.050	0.150 ± 0.050	1.200 ± 0.400	2.750 ± 1.150
1200	0.150 ± 0.050	0.250 ± 0.050	0.400 ± 0.100	0.650 ± 0.150*
2100	0.350 ± 0.050	0.550 ± 0.050	1.650 ± 0.250*	3.850 ± 0.550*
2400	0.400 ± 0.100	0.700 ± 0.000	2.350 ± 0.550	0.500 ± 0.000
0300	0.400 ± 0.000	0.500 ± 0.100	0.800 ± 0.200	2.300 ± 0.700*

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* Significantly different from the control group ($P \le 0.05$) by Shirley's test

[a] Mean ± standard error

[b] Both samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

[c] n=1

TABLE I2
Plasma Nitrite and Blood Methemoglobin Concentrations at 2 Weeks in Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite[a]

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Plasma Nitrite (µg/mL)				
Time of collection				
0600	—[b]	—	7.400[c]	9.700 ± 2.300
1200	—	—	—	—
2100	—	—	3.500[c]	3.450 ± 1.350
2400	—	—	—	4.086 ± 0.079
0300	—	—	2.900[c]	6.600[c]
Blood Methemoglobin (g/dL)				
Time of collection				
0600	0.250 ± 0.050	0.250 ± 0.150	1.650 ± 0.050	6.350 ± 1.550
1200	0.250 ± 0.050	0.200 ± 0.100	0.200 ± 0.100	0.600 ± 0.200
2100	0.350 ± 0.050	0.600 ± 0.200	2.650 ± 0.150*	3.850 ± 0.750*
2400	0.500 ± 0.000	0.500 ± 0.000	1.900 ± 0.400	4.350 ± 1.050*
0300	0.400 ± 0.100	0.700 ± 0.100	2.250 ± 0.450	3.850 ± 2.550

---

* Significantly different from the control group ($P \leq 0.05$) by Shirley's test

[a] Mean ± standard error

[b] Both samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

[c] n=1

TABLE I3
Plasma Nitrite and Blood Methemoglobin Concentrations at 3 Months in Male Rats in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Plasma Nitrite (µg/mL)				
Time of collection				
0600	—[b]	—	—	1.800[c]
1200	—	—	—	4.200[c]
2100	—	—	2.000[c]	3.000[c]
2400	—	—	—	4.500 ± 1.100
0300	—	—	—	—
Blood Methemoglobin (g/dL)				
Time of collection				
0600	0.700 ± 0.000	0.750 ± 0.050	2.100 ± 0.700	4.400 ± 1.900*
1200	0.300 ± 0.000	0.300 ± 0.000	0.400 ± 0.000	2.450 ± 0.050*
2100	0.350 ± 0.050	0.600 ± 0.000	1.450 ± 0.250	1.350 ± 0.750
2400	0.450 ± 0.050	0.600 ± 0.000	0.900 ± 0.100*	3.400 ± 0.600*
0300	0.300 ± 0.000	0.400 ± 0.000	0.650 ± 0.350	1.150 ± 0.750

* Significantly different from the control group ($P \leq 0.05$) by Shirley's test

[a] Mean ± standard error

[b] Both samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

[c] n=1

TABLE I4
Plasma Nitrite and Blood Methemoglobin Concentrations at 3 Months in Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Plasma Nitrite (μg/mL)				
Time of collection				
0600	—[b]	—	—	2.400[c]
1200	—	—	—	—
2100	—	—	—	3.000[c]
1200	—	—	—	—
0300	—	—	—	2.700[c]
Blood Methemoglobin (g/dL)				
Time of collection				
0600	0.450 ± 0.050	0.500 ± 0.100	0.550 ± 0.050	3.550 ± 1.050
1200	0.150 ± 0.050	0.300 ± 0.000	0.400 ± 0.000*	0.900 ± 0.400*
2100	0.200 ± 0.000	0.650 ± 0.250	3.650 ± 0.550*	2.250 ± 0.050
2400	0.350 ± 0.150	0.450 ± 0.150	1.150 ± 0.550	2.400 ± 1.200
0300	0.300 ± 0.100	0.450 ± 0.050	1.600 ± 0.300	3.000 ± 2.300

---

* Significantly different from the control group ($P \leq 0.05$) by Dunn's or Shirley's test

[a] Mean ± standard error

[b] Both samples in this group were below the estimated limit of quantitation (1.7 μg/mL).

[c] n=1

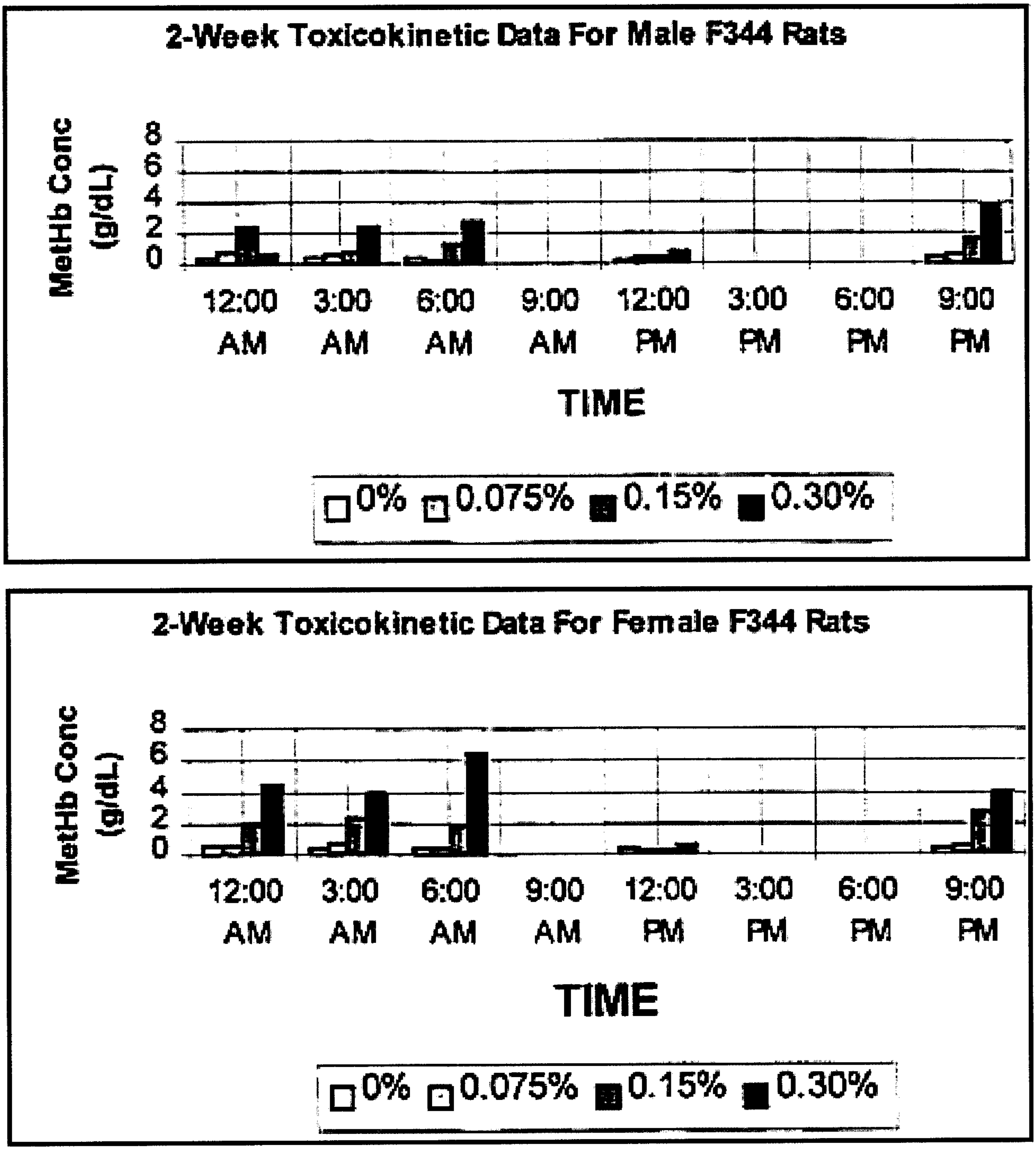

FIGURE 11
Blood Methemoglobin Concentrations at 2 Weeks in Male and Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite
(0% = 0 ppm, 0.075% = 750 ppm, 0.15% = 1,500 ppm, 0.30% = 3,000 ppm)

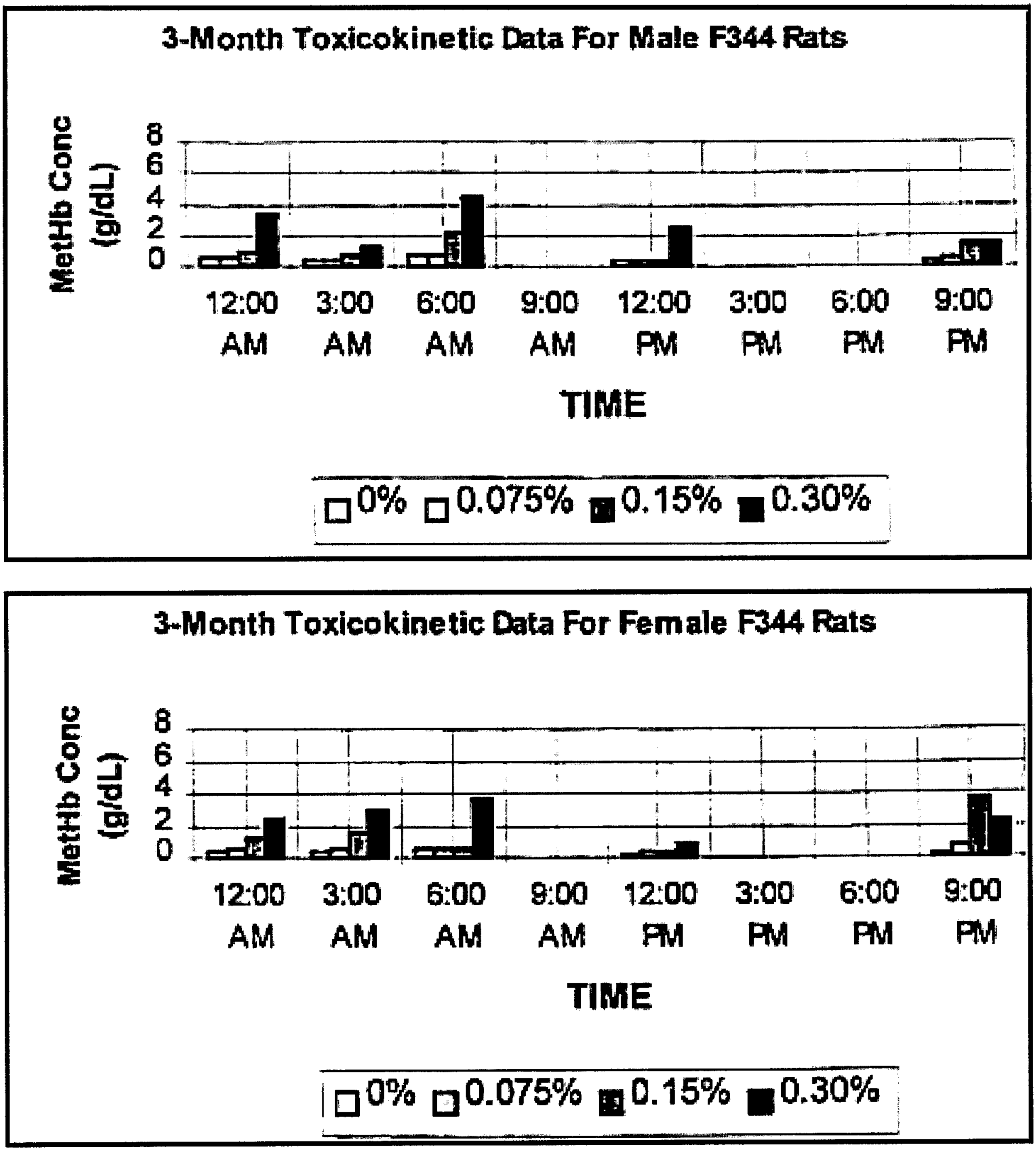

FIGURE 12
Blood Methemoglobin Concentrations at 3 Months in Male and Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite
(0% = 0 ppm, 0.075% = 750 ppm, 0.15% = 1,500 ppm, 0.30% = 3,000 ppm)

TABLE I5
Plasma Nitrite and Blood Methemoglobin Concentrations at 18 Months in Aged Male Rats after a Single Gavage Dose of 40 mg/kg Sodium Nitrite[a]

	Time after Dosing (minutes)	Concentration
Plasma Nitrite (µg/mL)		
	2	—[b]
	5	2.500[c]
	10	8.000
	30	8.950 ± 2.050
	60	6.300
Methemoglobin (g/dL)		
	2	1.333 ± 0.176
	5	2.167 ± 0.328
	10	2.700 ± 0.208
	30	4.200 ± 0.300
	60	5.100 ± 1.100

---

[a] Mean ± standard error. Three animals were bled at 2, 5, and 10 minutes and two animals were bled at 30 and 60 minutes for blood methemoglobin measurements.

[b] All samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

[c] Two samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

TABLE I6
Plasma Nitrite and Blood Methemoglobin Concentrations at 18 Months in Aged Female Rats after a Single Gavage Dose of 40 mg/kg Sodium Nitrite[a]

	Time after Dosing (minutes)	Concentration
Plasma Nitrite (µg/mL)		
	2	—[b]
	5	3.500 ± 0.200
	10	6.067 ± 0.578
	30	8.133 ± 0.561
	60	6.350 ± 0.550
Methemoglobin (g/dL)		
	2	1.333 ± 0.203
	5	2.933 ± 0.145
	10	2.700 ± 0.265
	30	5.200 ± 0.058
	60	5.650 ± 0.150

---

[a] Mean ± standard error. Three animals were bled at 2, 5, 10, and 30 minutes and two animals were bled at 60 minutes for blood methemoglobin measurements.

[b] All samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

TABLE I17
Plasma Nitrite and Blood Methemoglobin Concentrations at 12 Months in Male Mice in the 2-Year Drinking Water Study of Sodium Nitrite[a]

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Plasma Nitrite (µg/mL)				
Time of collection				
0600	—[b]	—	2.140[c]	4.251 ± 1.095
1200	—	—	—	—
2100	—	—	2.329[c]	1.883[c]
2400	—	—	2.515[c]	17.111 ± 14.689
0300	—	—	—	2.370[c]
Blood Methemoglobin (g/dL)				
Time of collection				
0600	0.200 ± 0.000	0.200 ± 0.000	0.300 ± 0.000	0.750 ± 0.050
1200	0.200 ± 0.000	0.200 ± 0.000	0.200 ± 0.000	0.250 ± 0.050
2100	0.100 ± 0.000	0.150 ± 0.050	0.250 ± 0.050	0.200 ± 0.000
2400	0.300 ± 0.000	0.300 ± 0.100	0.200 ± 0.000	0.450 ± 0.050
0300	0.250 ± 0.050	0.200 ± 0.000	0.200 ± 0.000	0.300 ± 0.100

[a] Mean ± standard error

[b] Both samples in this group were below the estimated limit of quantitation (1.7 µg/mL).

[c] n=1

TABLE I18
Blood Methemoglobin Concentrations at 12 Months in Female Mice in the 2-Year Drinking Water Study of Sodium Nitrite[a]

	0 ppm	750 ppm	1,500 ppm	3,000 ppm
n	2	2	2	2
Time of collection				
0600	0.200 ± 0.000	0.200 ± 0.000	0.200 ± 0.100	0.250 ± 0.050
1200	0.400[b]	0.300 ± 0.000	0.300 ± 0.000	0.300 ± 0.000
2100	0.200 ± 0.000	0.200 ± 0.000	0.200 ± 0.000	0.200 ± 0.000
2400	0.150 ± 0.050	0.250 ± 0.050	0.250 ± 0.050	0.250 ± 0.050
0300	0.200 ± 0.000	0.300 ± 0.100	0.200 ± 0.000	0.250 ± 0.050

[a] Data are given in g/dL as mean ± standard error. All plasma nitrite samples were below the estimated limit of quantitation (1.7 µg/mL).

[b] n=1

TABLE I9
Plasma Nitrite and Blood Methemoglobin Concentrations at 18 Months in Aged Male Mice after a Single Gavage Dose of 62.5 mg/kg Sodium Nitrite[a]

	Time after Dosing (minutes)	Concentration
Plasma Nitrite (µg/mL)		
	2	22.233 ± 6.082
	5	23.967 ± 5.206
	10	32.267 ± 4.018
	30	13.533 ± 0.717
	60	8.933 ± 0.921
Methemoglobin (g/dL)		
	2	1.100 ± 0.208
	5	1.833 ± 0.088
	10	2.000 ± 0.289
	30	1.600 ± 0.058
	60	0.800 ± 0.153

[a] Mean ± standard error. Three animals were bled at each time point.

TABLE I10
Plasma Nitrite and Blood Methemoglobin Concentrations at 18 Months in Aged Female Mice after a Single Gavage Dose of 62.5 mg/kg of Sodium Nitrite[a]

	Time after Dosing (minutes)	Concentration
Plasma Nitrite (µg/mL)		
	2	27.100 ± 4.637
	5	64.933 ± 6.199
	10	50.800 ± 3.293
	30	45.933 ± 6.583
	60	43.967 ± 11.737
Methemoglobin (g/dL)		
	2	1.533 ± 0.376
	5	2.900 ± 0.361
	10	3.433 ± 0.393
	30	3.833 ± 0.857
	60	5.000 ± 0.231

[a] Mean ± standard error. Three animals were bled at each time point.

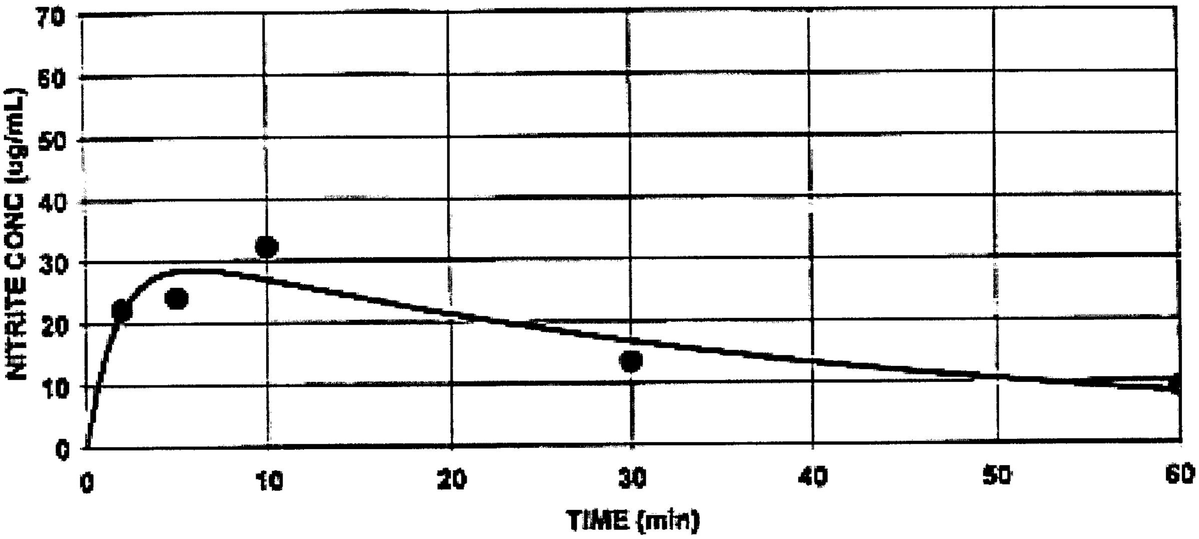

FIGURE 13
Plasma Nitrite Concentrations at 18 Months in Aged Male Mice after a Single Gavage Dose of 62.5 mg/kg Sodium Nitrite

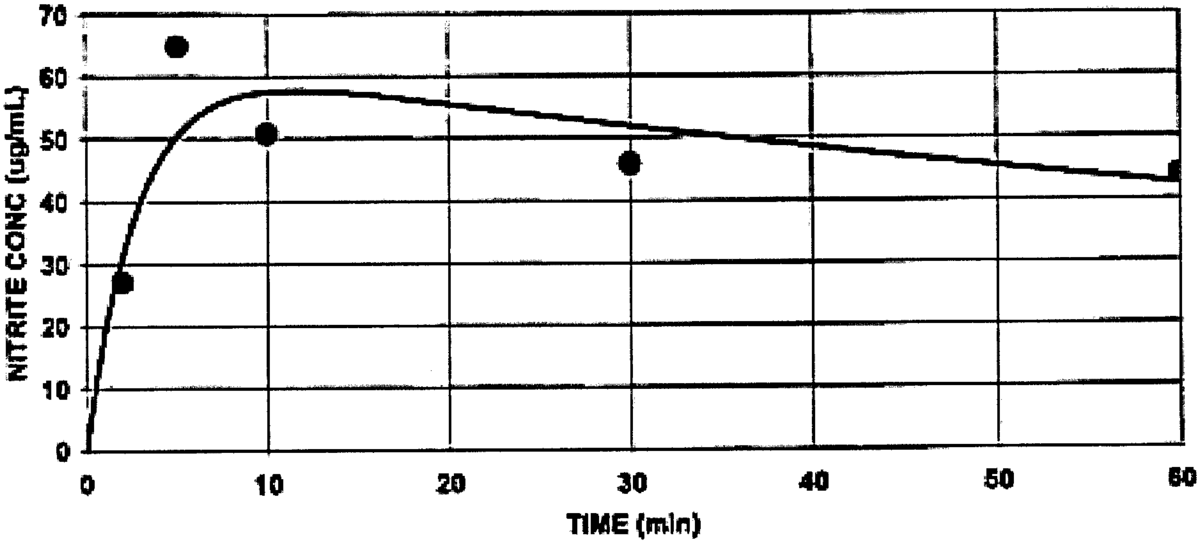

FIGURE 14
Plasma Nitrite Concentrations at 18 Months in Aged Female Mice after a Single Gavage Dose of 62.5 mg/kg Sodium Nitrite

APPENDIX J
CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

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CHEMICAL CHARACTERIZATION AND DOSE FORMULATION STUDIES

PROCUREMENT AND CHARACTERIZATION OF SODIUM NITRITE

Sodium nitrite was obtained from J.T. Baker, Inc. (Phillipsburg, NJ), in two lots (A42340 and H05714). Lot A42340 was used during the 14-week and 2-year studies and lot H05714 was used during the 2-year studies. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory, Midwest Research Institute (Kansas City, MO). Reports on analyses performed in support of the sodium nitrite studies are on file at the National Institute of Environmental Health Sciences.

Both lots of the chemical, a white crystalline solid, were identified as sodium nitrite by infrared and ultraviolet/visible spectroscopy. The infrared spectra were consistent with the literature spectra (*Sadtler Standard Spectra*; *Aldrich*, 1985) of sodium nitrite; the ultraviolet/visible spectra were consistent with the structure of sodium nitrite. The infrared spectrum is presented in Figure J1. The melting point ranges of 281.2° to 282.7° C determined for lot A42340 and 281.1° to 281.6° C for lot H05714 were in agreement with a value of 281° C cited in the literature (Klein and McDonald, 1982).

The purity of both lots was determined by elemental analyses, weight loss on drying, spark source mass spectrometry, and high-performance liquid chromatography (HPLC). The purity of lot A42340 was also analyzed by differential scanning calorimetry, a United States Pharmacopeia (USP) XXI titrimetric assay, and American Chemical Society (ACS) tests for chloride and sulfate. Elemental analyses of lot A42340 were performed by the analytical chemistry laboratory and Lancaster Laboratories (Lancaster, PA); elemental analyses of lot H05714 were performed by Galbraith Laboratories, Inc. (Knoxville, TN). Spark source mass spectrometry was performed by the analytical chemistry laboratory for lot A42340 and by Accu-Labs Research, Inc. (Golden, CO), for lot H05714. Lot A42340 was analyzed with HPLC by system A, and lot H05714 was analyzed with HPLC by system B (Table J1).

For lot A42340, elemental analyses for sodium and nitrogen were in agreement with the theoretical values for sodium nitrite. Weight loss on drying indicated 0.006% ± 0.002% water. Differential scanning calorimetry determined a melting point of 280.8° C and indicated that the purity was greater than 99 molar percent. Spark source mass spectrometry identified sodium as the major component; the concentration of impurities was less than 87 ppm. The USP method of titration with potassium permanganate to a colorimetric endpoint indicated a purity of 100.4% ± 0.4%. ACS tests on lot A42340 for chloride and sulfate produced less turbidity than tests on a standard solution, indicating less than 0.005% chloride ion and less than 0.01% sulfate ion. HPLC indicated a major peak and a single impurity with an area of 0.2% relative to the major peak area (system A). The cumulative data indicated a purity greater than 99%.

For lot H05714, elemental analyses for sodium and nitrogen were in agreement with the theoretical values for sodium nitrite. There was no weight loss on drying. HPLC by system B indicated one major peak and one impurity with an area of 0.15% relative to the major peak area. Spark source mass spectrometry identified sodium as the major component and no major contaminants; traces of calcium, fluorine, potassium, phosphorus, and iron were detected. The overall purity of lot H05714 was determined to be 99% or greater.

Stability studies of the bulk chemical were performed by the analytical chemistry laboratory. Permanganate titration was performed by reacting sodium nitrite with an excess of standard potassium permanganate (USP XIX). These studies indicated that sodium nitrite was stable as a bulk chemical for 2 weeks when stored protected from light at temperatures up to 60° C.

To ensure stability, the bulk chemical was stored at 5° C, protected from light, in closed containers under an inert atmospere during the 14-week study and in Nalgene® containers at room temperature, protected from light, during the 2-year studies. Stability was monitored by the study laboratory with infrared and ultraviolet/visible spectroscopy and HPLC by system C (14-week studies) and with ultraviolet spectroscopy (2-year studies). No degradation of the bulk chemical was detected.

PREPARATION AND ANALYSIS OF DOSE FORMULATIONS

The dose formulations were prepared every 2 weeks (14-week studies) or approximately every 4 weeks (2-year studies) by mixing sodium nitrite with water (Table J2). Formulations were stored in glass carboys at room temperature for up to 3 weeks for the 14-week studies and in Nalgene® tanks at room temperature for up to 35 days for the 2-year studies.

Stability studies of a 0.075 mg/mL dose formulation were performed by the analytical chemistry laboratory using ultraviolet/visible spectrophotometry by measuring absorbance at 347 nm of an aliquot of the sample treated with a salt solution (sodium sulfate and sodium acetate) and a color reagent (hydrochloric acid, resorcinol and zinconyl chloride). Stability was confirmed for at least 35 days for dose formulations stored at 5° C or at room temperature in the dark.

Periodic analyses of the dose formulations of sodium nitrite were conducted by the study laboratory using ultraviolet/visible spectroscopy as described for the stability study. During the 14-week studies, the dose formulations were analyzed at the beginning, midpoint, and end of the studies; animal room samples of these dose formulations were also analyzed (Table J3). All 32 dose formulations analyzed were within 10% of the target concentrations; all 30 animal room samples for rats and 22 of 25 for mice were also within 10% of the target concentrations. During the 2-year studies, the dose formulations were analyzed approximately every 8 to 12 weeks; animal room samples were also analyzed periodically (Table J4). All 39 of the 2-year dose formulations analyzed were within 10% of the target concentrations. All 12 animal room samples for rats and for mice were within 10% of the target concentrations. Results of periodic referee analyses performed by the analytical chemistry laboratory agreed with the results obtained by the study laboratory (Table J5).

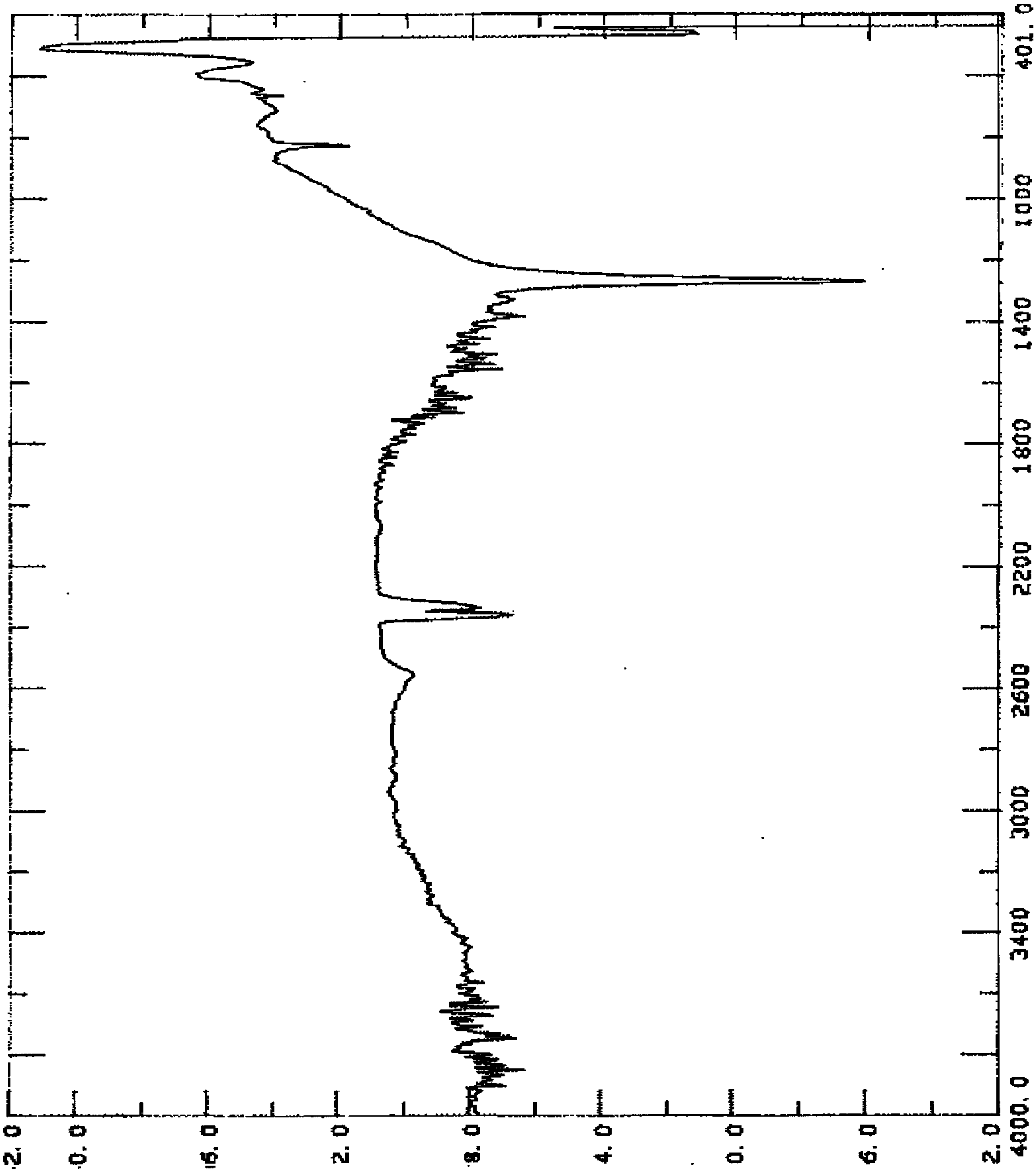

FIGURE J1
Infrared Absorption Spectrum of Sodium Nitrite

TABLE J1
High-Performance Liquid Chromatography Systems Used in the Drinking Water Studies of Sodium Nitrite[a]

Detection System	Column	Solvent System
System A		
Ultraviolet (230 nm)	Whatman Partisil 10 SAX, 250 mm × 4.6 mm (Whatman, Inc., Clifton, NJ)	0.0125 M potassium dihydrogen phosphate, pH 4.5; flow rate 1.0 mL/minute
System B		
Dionex CDM-3 Conductivity Detector (300 µS)	Dionex AS4A-SC, 250 mm × 4.0 mm, 13 µm (Dionex Corp., Sunnyvale, CA)	1.5 mM sodium carbonate/1.2 mM sodium bicarbonate, in water; flow rate 2 mL/minute
System C		
Waters Model 430 Conductivity Detector	Waters IC-PAK Anion, 5 cm × 4.6 mm (Waters-Millipore, Milford, MA)	Water containing (in 1 L) approximately 4.3 mL glycerin, 20 mL n-butanol, 120 mL acetonitrile, 0.3 g sodium gluconate, 0.3 g boric acid, and 0.4 g sodium tetraborate decahydrate; flow rate 0.9 mL/minute

[a] Chromatographs were manufactured by Waters-Millipore (Milford, MA) and Dionex Corp. (Sunnyvale, CA).

TABLE J2
Preparation and Storage of Dose Formulations in the Drinking Water Studies of Sodium Nitrite

14-Week Studies	2-Year Studies
Preparation	
Sodium nitrite was mixed with charcoal-filtered deionized water (pH 6.8-7.0) with a stir-bar and then further diluted with water. Dose formulations were prepared every 2 weeks.	Sodium nitrite was mixed with tap water with an automatic stirrer, then further diluted and stirred for an additional 5 minutes. A 2-L portion was removed from the bottom of the preparation and returned to the top; the formulation was then stirred for 5 minutes. Dose formulations were prepared approximately every 4 weeks.
Chemical Lot Number	
Lot A42340	Lots A42340 and H05714
Maximum Storage Time	
3 weeks	35 days
Storage Conditions	
Stored in glass carboys at room temperature	Stored in Nalgene® tanks at room temperature
Study Laboratory	
Microbiological Associates, Inc. (Bethesda, MD)	Battelle Columbus Laboratories (Columbus, OH)
Referee Laboratory	
Midwest Research Institute (Kansas City, MO)	None

Table J3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 14-Week Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration[a] (%)	Difference from Target (%)
Rats				
1 August 1989	3 August 1989	0.0375	0.0361	-4
		0.0375	0.0358	-5
		0.075	0.0726	-3
		0.075	0.0724	-3
		0.15	0.139	-7
		0.15	0.159	+6
		0.15	0.137	-9
		0.30	0.286	-5
		0.30	0.295	-2
		0.50	0.532	+6
		0.50	0.541	+8
		0.50	0.534	+7
	24 August 1989[b]	0.0375	0.0349	-7
		0.0375	0.0342	-9
		0.075	0.0698	-7
		0.075	0.0708	-6
		0.15	0.139	-7
		0.15	0.139	-7
		0.30	0.273	-9
		0.30	0.291	-3
		0.50	0.500	0
		0.50	0.490	-2
12 September 1989	13 September 1989	0.0375	0.0384	+2
		0.0375	0.0378	+1
		0.075	0.0768	+2
		0.075	0.0770	+3
		0.15	0.153	+2
		0.15	0.155	+3
		0.30	0.310	+3
		0.30	0.311	+4
		0.50	0.524	+5
		0.50	0.513	+3
	5 October 1989[b]	0.0375	0.0389	+4
		0.0375	0.0386	+3
		0.075	0.0776	+3
		0.075	0.0763	+2
		0.15	0.155	+3
		0.15	0.164	+9
		0.30	0.307	+2
		0.30	0.316	+5
		0.50	0.523	+5
		0.50	0.523	+5

TABLE J3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 14-Week Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration (%)	Difference from Target (%)
24 October 1989	25 October 1989	0.0375	0.0403	+7
		0.0375	0.0405	+8
		0.075	0.0828	+10
		0.075	0.0779	+4
		0.15	0.162	+8
		0.15	0.162	+8
		0.30	0.330	+10
		0.30	0.327	+9
		0.50	0.528	+6
		0.50	0.525	+5
	15 November 1989[b]	0.0375	0.0397	+6
		0.0375	0.0403	+7
		0.075	0.0763	+2
		0.075	0.0786	+5
		0.15	0.162	+8
		0.15	0.159	+6
		0.30	0.318	+6
		0.30	0.307	+2
		0.50	0.520	+4
		0.50	0.512	+2
Mice				
1 August 1989	3 August 1989	0.0375	0.0361	-4
		0.0375	0.0358	-5
		0.075	0.0726	-3
		0.075	0.0724	-3
		0.15	0.139	-7
		0.15	0.159	+6
		0.15	0.137	-9
		0.30	0.286	-5
		0.30	0.295	-2
		0.50	0.532	+6
		0.50	0.541	+8
		0.50	0.534	+7
	24 August 1989[b]	0.0375	0.0348	-7
		0.0375	0.0340	-9
		0.075	0.0714	-5
		0.075	0.0710	-5
		0.15	0.138	-8
		0.15	0.156	+4
		0.30	0.282	-6
		0.30	0.282	-6
		0.50	0.478	-4
		0.50	0.500	0

TABLE J3
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 14-Week Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration (%)	Difference from Target (%)
12 September 1989	13 September 1989	0.0375	0.0384	+2
		0.0375	0.0378	+1
		0.075	0.0768	+2
		0.075	0.0770	+3
		0.15	0.153	+2
		0.15	0.155	+3
		0.30	0.310	+3
		0.30	0.311	+4
		0.50	0.524	+5
		0.50	0.513	+3
	5 October 1989[b]	0.0375	0.0303	-19
		0.0375	0.0556	+48
		0.075	0.0590	-21
		0.075	0.0771	+3
		0.15	0.160	+7
		0.15	0.161	+7
		0.30	0.280	-7
		0.30	0.307	+2
		0.50	0.535	+7
		0.50	0.516	+3
	11 October 1989[b]	0.0375	0.0282	-25
		0.0375	0.0537	+43
		0.075	0.0598	-20
24 October 1989	25 October 1989	0.0375	0.0403	+7
		0.0375	0.0405	+8
		0.075	0.0828	+10
		0.075	0.0779	+4
		0.15	0.162	+8
		0.15	0.162	+8
		0.30	0.330	+10
		0.30	0.327	+9
		0.50	0.528	+6
		0.50	0.525	+5
	15 November 1989[b]	0.0375	0.0376	0
		0.075	0.0781	+4
		0.15	0.160	+7
		0.30	0.318	+6
		0.50	0.471	-6

---

[a] Results of duplicate analyses

[b] Animal room samples

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration[a] (%)	Difference from Target (%)
Rats				
1 August 1995	3 August 1995	0.075	0.07639	+2
		0.15	0.1534	+2
		0.30	0.3062	+2
	22 August 1995[b]	0.075	0.07438	-1
		0.15	0.1530	+2
		0.30	0.3047	+2
20 September 1995	21-22 September 1995	0.075	0.07480	0
		0.15	0.1548	+3
		0.30	0.2928	-2
13 December 1995	14 December 1995	0.075	0.07819	+4
		0.15	0.1577	+5
		0.30	0.3154	+5
7 February 1996	7 February 1996	0.075	0.07550	+1
		0.15	0.1524	+2
		0.30	0.3027	+1
	15 March 1996[b]	0.075	0.07495	0
		0.15	0.1484	-1
		0.30	0.2951	-2
3 April 1996	3 April 1996	0.075	0.07803	+4
		0.15	0.1578	+5
		0.30	0.3148	+5
26 June 1996	26 June 1996	0.075	0.07964	+6
		0.15	0.1572	+5
		0.30	0.3057	+2
21 August 1996	22 August 1996	0.075	0.07635	+2
		0.15	0.1518	+1
		0.30	0.3049	+2
	30 September 1996[b]	0.075	0.07991	+7
		0.15	0.1563	+4
		0.30	0.3127	+4
16 October 1996	16 October 1996	0.075	0.07684	+2
		0.15	0.1491	-1
		0.30	0.3125	+4
8 January 1997	9 January 1997	0.075	0.07751	+3
		0.15	0.1546	+3
		0.30	0.3083	+3

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration (%)	Difference from Target (%)
5 March 1997	6 March 1997	0.075	0.07682	+2
		0.15	0.1535	+2
		0.30	0.3057	+2
	15 April 1997[b]	0.075	0.08139	+9
		0.15	0.1547	+3
		0.30	0.3112	+4
30 April 1997	1 May 1997	0.075	0.07732	+3
		0.15	0.1537	+2
		0.30	0.3031	+1
23 July 1997	23 July 1997	0.075	0.07385	-2
		0.15	0.1561	+4
		0.30	0.2984	-1
Mice				
25 July 1995	26 July 1995	0.075	0.07694	+3
		0.15	0.1551	+3
		0.30	0.3035	+1
	9 August 1995[b]	0.075	0.07705	+3
		0.15	0.1550	+3
		0.30	0.3040	+1
1 August 1995	3 August 1995	0.075	0.07639	+2
		0.15	0.1534	+2
		0.30	0.3062	+2
20 September 1995	21-22 September 1995	0.075	0.07480	0
		0.15	0.1548	+3
		0.30	0.2928	-2
13 December 1995	14 December 1995	0.075	0.07819	+4
		0.15	0.1577	+5
		0.30	0.3154	+5
7 February 1996	7 February 1996	0.075	0.07550	+1
		0.15	0.1524	+2
		0.30	0.3027	+1
	15 March 1996[b]	0.075	0.07319	-2
		0.15	0.1450	-3
		0.30	0.2891	-4

TABLE J4
Results of Analyses of Dose Formulations Administered to Rats and Mice in the 2-Year Drinking Water Studies of Sodium Nitrite

Date Prepared	Date Analyzed	Target Concentration (%)	Determined Concentration (%)	Difference from Target (%)
3 April 1996	3 April 1996	0.075	0.07803	+4
		0.15	0.1578	+5
		0.30	0.3148	+5
26 June 1996	26 June 1996	0.075	0.07964	+6
		0.15	0.1572	+5
		0.30	0.3057	+2
21 August 1996	22 August 1996	0.075	0.07635	+2
		0.15	0.1518	+1
		0.30	0.3049	+2
	30 September 1996[b]	0.075	0.08076	+8
		0.15	0.1583	+6
		0.30	0.3081	+3
16 October 1996	16 October 1996	0.075	0.07684	+2
		0.15	0.1491	-1
		0.30	0.3125	+4
8 January 1997	9 January 1997	0.075	0.07751	+3
		0.15	0.1546	+3
		0.30	0.3083	+3
5 March 1997	6 March 1997	0.075	0.07682	+2
		0.15	0.1535	+2
		0.30	0.3057	+2
	15 April 1997[b]	0.075	0.07688	+3
		0.15	0.1534	+2
		0.30	0.3109	+4
30 April 1997	1 May 1997	0.075	0.07732	+3
		0.15	0.1537	+2
		0.30	0.3031	+1
23 July 1997	23 July 1997	0.075	0.07385	-2
		0.15	0.1561	+4
		0.30	0.2984	-1

[a] Results of duplicate analyses
[b] Animal room samples

TABLE J5
Results of Referee Analyses of Dose Formulations Administered to Rats and Mice in the 14-Week Drinking Water Studies of Sodium Nitrite

Date Prepared	Target Concentration (%)	Determined Concentration (%) Study Laboratory[a]	Determined Concentration (%) Referee Laboratory[b]
1 August 1989	0.3	0.286	0.282 ± 0.000
12 September 1989	0.075	0.0768	0.0788 ± 0.0002

[a] Results of duplicate analyses

[b] Results of triplicate analyses (mean ± standard error)

APPENDIX K
WATER AND COMPOUND CONSUMPTION IN THE 2-YEAR DRINKING WATER STUDIES OF SODIUM NITRITE

TABLE K1 **Water and Compound Consumption by Male Rats in the 2-Year Drinking Water Study of Sodium Nitrite** **262**

TABLE K2 **Water and Compound Consumption by Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite** **263**

TABLE K3 **Water and Compound Consumption by Male Mice in the 2-Year Drinking Water Study of Sodium Nitrite** **264**

TABLE K4 **Water and Compound Consumption by Female Mice in the 2-Year Drinking Water Study of Sodium Nitrite** **265**

TABLE K1
Water and Compound Consumption by Male Rats in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm		750 ppm			1,500 ppm			3,000 ppm		
Week	Water (g/day)[a]	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
1	16.4	107	17.8	107	125	18.1	107	256	13.4	107	375
2	17.0	144	17.7	144	92	18.2	143	192	14.7	134	329
6	19.3	259	18.9	254	56	18.0	254	106	15.9	242	197
10	18.9	314	17.7	307	43	16.9	304	84	13.6	288	142
14	19.5	354	18.0	348	39	16.3	341	72	14.2	322	133
18	18.3	381	16.7	376	33	15.3	367	63	13.5	349	116
22	18.0	406	17.6	399	33	17.1	392	66	14.9	369	121
26	17.9	426	17.5	416	32	16.9	410	62	15.1	385	118
30	17.4	438	17.2	435	30	16.2	423	57	15.0	400	112
34	18.9	450	17.5	446	29	17.9	435	62	16.0	412	116
38	18.5	458	17.0	460	28	16.8	443	57	15.7	418	113
42	17.4	466	16.6	462	27	16.5	450	55	15.4	422	109
46	17.2	478	16.1	474	26	16.1	463	52	15.2	433	105
50	17.2	487	16.3	486	25	16.5	470	53	14.9	437	102
54	17.5	492	16.2	487	25	16.7	473	53	14.5	441	98
58	16.9	491	16.6	495	25	16.6	477	52	14.9	445	101
62	17.7	499	16.8	495	25	17.0	481	53	15.2	448	102
66	17.7	499	16.6	494	25	16.9	480	53	15.9	444	107
70	17.9	499	17.0	499	25	16.9	481	53	16.3	452	108
74	18.4	501	17.8	498	27	17.6	477	55	16.2	449	109
78	18.1	501	17.7	497	27	18.0	481	56	15.5	446	104
82	18.5	502	17.5	493	27	17.5	479	55	16.1	445	108
86	17.5	497	17.3	495	26	17.5	481	55	15.4	445	104
90	17.7	494	16.8	499	25	17.1	480	54	14.5	441	98
94	17.6	495	16.8	496	25	17.2	481	54	14.6	440	100
98	17.5	492	16.2	488	25	16.8	480	52	14.4	442	98
102	17.9	496	16.7	485	26	18.0	483	56	15.0	441	102
Mean for weeks											
1-13	17.9	206	18.0	203	79	17.8	202	159	14.4	193	261
14-52	18.0	435	17.1	430	30	16.6	420	60	15.0	395	115
53-102	17.8	497	16.9	494	26	17.2	480	54	15.3	445	103

[a] Grams of water consumed per animal per day

[b] Milligrams of sodium nitrite consumed per kilogram body weight per day

TABLE K2
Water and Compound Consumption by Female Rats in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm		750 ppm			1,500 ppm			3,000 ppm		
Week	Water (g/day)[a]	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
1	13.1	96	13.0	95	103	13.7	96	215	12.0	95	377
2	14.9	118	14.4	116	94	14.3	115	186	11.9	111	324
6	14.7	168	15.0	164	68	13.4	164	123	11.0	159	207
10	13.9	187	13.2	182	55	12.2	183	101	10.0	177	169
14	12.8	201	12.2	196	46	11.4	195	88	9.3	188	148
18	13.0	212	12.2	206	45	11.4	204	84	9.7	197	147
22	13.6	220	12.7	214	45	11.7	212	83	10.5	204	155
26	13.5	227	12.2	221	41	12.0	218	83	10.2	211	145
30	12.2	238	11.3	231	37	11.0	227	73	9.6	217	133
34	12.7	245	11.9	236	38	11.5	234	74	10.1	224	136
38	12.6	254	11.8	245	36	11.2	241	70	10.2	231	132
42	12.6	260	11.6	251	35	11.2	246	68	10.1	235	129
46	12.1	271	11.4	259	33	10.8	252	65	9.8	241	122
50	11.9	281	11.2	269	31	11.1	264	63	10.2	250	122
54	12.0	286	11.2	276	31	11.2	270	62	10.1	256	119
58	11.8	293	11.8	284	31	11.4	276	62	10.1	261	116
62	11.9	302	11.8	291	30	11.5	287	60	11.7	265	132
66	12.9	307	12.1	293	31	12.0	288	63	11.3	269	126
70	13.1	315	12.8	305	31	12.5	299	63	11.7	276	127
74	13.4	319	13.1	312	31	13.0	305	64	11.6	279	125
78	13.5	329	13.5	319	32	13.4	313	64	12.0	285	126
82	13.6	332	12.9	320	30	12.8	314	61	11.6	287	121
86	13.5	333	13.7	325	32	13.3	319	62	12.1	291	125
90	13.5	339	13.1	327	30	12.6	323	58	11.3	290	117
94	14.1	346	13.4	330	31	12.7	326	58	12.2	302	121
98	14.8	350	13.6	333	31	13.2	332	60	12.1	308	118
102	14.4	344	14.4	337	32	13.6	332	61	12.6	312	121
Mean for weeks											
1-13	14.1	142	13.9	139	80	13.4	139	156	11.2	135	269
14-52	12.7	241	11.8	233	39	11.3	229	75	10.0	220	137
53-102	13.3	323	12.9	312	31	12.5	307	61	11.6	283	123

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[a] Grams of water consumed per animal per day

[b] Milligrams of sodium nitrite consumed per kilogram body weight per day

TABLE K3
Water and Compound Consumption by Male Mice in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm		750 ppm			1,500 ppm			3,000 ppm		
Week	Water (g/day)[a]	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
2	3.2	23.7	3.4	23.7	106	3.3	23.6	211	2.9	23.4	368
6	3.1	29.5	3.1	29.6	78	3.2	29.1	164	2.7	28.3	288
10	3.1	34.9	3.2	34.5	69	3.0	33.7	133	2.7	32.7	247
14	3.2	39.1	3.1	38.9	60	3.1	37.7	124	2.9	36.2	244
18	3.8	43.2	3.5	42.9	62	3.4	41.9	123	3.3	40.2	247
22	3.3	45.6	3.1	44.9	52	3.1	44.0	106	2.9	42.1	209
26	3.6	47.5	3.3	46.6	53	3.1	45.7	102	3.0	44.6	203
30	3.3	49.6	3.2	48.9	48	3.1	48.3	96	3.0	46.9	195
34	3.4	50.8	3.1	50.1	47	3.1	49.2	95	3.0	48.4	188
38	3.6	50.6	3.2	49.7	49	3.3	48.7	101	3.1	48.1	193
42	3.7	50.9	3.4	49.9	51	3.2	48.4	101	3.1	48.6	191
46	3.8	50.8	3.3	49.4	50	3.3	48.3	102	3.0	48.6	186
50	3.9	50.3	3.7	49.0	57	3.4	48.0	107	3.1	48.3	195
54	3.8	51.3	3.4	50.5	50	3.3	49.2	102	3.0	49.5	180
58	3.7	51.1	3.3	49.6	50	3.5	49.3	105	3.2	49.8	194
62	3.7	51.4	3.5	50.4	52	3.4	49.4	103	3.1	49.5	189
66	3.8	50.7	3.5	49.8	53	3.6	49.0	109	3.2	48.8	200
70	3.9	50.0	3.6	49.7	55	3.6	48.8	110	3.3	48.7	206
73	3.8	49.4	3.6	49.5	54	3.7	48.8	114	3.3	48.1	207
78	4.1	49.4	3.8	49.1	58	3.9	48.2	120	3.5	47.6	222
82	4.0	48.7	3.8	48.4	59	4.0	48.2	125	3.5	46.6	223
86	4.1	47.2	3.9	46.9	63	3.9	46.8	124	3.6	45.4	235
90	3.8	46.4	3.5	46.4	57	3.7	46.1	120	3.2	44.7	216
94	4.0	44.7	3.4	44.4	57	3.7	44.5	123	3.3	44.1	221
98	3.7	43.0	3.5	43.7	61	3.6	43.6	126	3.1	42.8	221
102	4.0	40.2	3.6	41.3	65	3.6	41.4	131	3.3	41.0	244
Mean for weeks											
2-13	3.1	29.4	3.2	29.3	84	3.2	28.8	169	2.8	28.1	301
14-52	3.6	47.8	3.3	47.0	53	3.2	46.0	106	3.1	45.2	205
53-102	3.9	48.0	3.6	47.7	56	3.6	47.2	116	3.3	46.7	212

[a] Grams of water consumed per animal per day

[b] Milligrams of sodium nitrite consumed per kilogram body weight per day

TABLE K4
Water and Compound Consumption by Female Mice in the 2-Year Drinking Water Study of Sodium Nitrite

	0 ppm		750 ppm			1,500 ppm			3,000 ppm		
Week	Water (g/day)[a]	Body Weight (g)	Water (g/day)	Body Weight (g)	Dose (mg/kg)[b]	Water (g/day)	Body Weight (g)	Dose (mg/kg)	Water (g/day)	Body Weight (g)	Dose (mg/kg)
1	2.9	18.9	2.8	18.9	113	2.7	18.8	211	2.3	18.9	370
2	3.0	19.4	3.0	19.7	114	3.0	19.5	227	2.3	19.4	364
6	2.9	23.7	3.0	23.9	93	2.8	23.9	174	2.5	23.2	325
10	3.1	28.2	2.8	28.4	74	2.8	28.2	149	2.5	26.9	281
14	2.9	31.4	3.0	32.6	69	2.7	31.5	129	2.9	30.3	282
18	3.1	35.6	2.8	37.8	55	2.7	36.5	110	2.4	34.4	208
22	2.8	40.0	2.4	41.7	44	2.4	40.6	88	2.2	38.2	177
26	2.5	43.2	2.4	44.9	40	2.3	43.7	80	2.2	41.1	163
30	2.6	46.6	2.3	47.4	36	2.3	46.2	74	2.3	43.3	158
34	2.4	49.2	2.2	49.6	34	2.3	48.8	71	2.3	45.4	151
38	2.6	51.1	2.5	51.1	36	2.4	50.0	73	2.4	46.9	153
42	2.6	53.4	2.4	53.3	34	2.3	52.3	65	2.3	48.8	139
46	2.4	54.2	2.2	54.6	30	2.1	54.0	59	2.1	50.8	125
50	2.5	55.3	2.2	55.6	30	2.2	54.8	61	2.1	52.5	120
54	2.2	56.1	2.2	55.3	30	2.2	55.2	59	2.0	53.0	111
58	2.6	56.7	2.6	57.0	34	2.5	56.6	67	2.3	54.2	126
62	2.3	58.8	2.2	58.2	28	2.1	58.4	54	2.0	56.1	106
66	2.4	58.3	2.3	57.5	31	2.3	57.4	61	2.0	55.4	111
70	2.7	57.7	2.3	56.8	30	2.3	56.5	62	2.1	54.4	117
73	2.5	56.1	2.3	56.1	30	2.3	55.0	62	2.1	53.7	116
78	2.8	55.5	2.6	56.3	35	2.6	55.0	71	2.3	53.8	131
82	2.8	55.0	2.6	55.6	35	2.4	54.2	67	2.3	54.2	126
86	2.8	53.5	2.5	53.7	35	2.5	53.5	71	2.2	53.2	123
90	3.2	52.6	2.6	52.8	37	2.7	53.1	75	2.2	51.2	127
94	3.1	52.1	2.5	52.1	36	2.6	52.3	76	2.1	49.6	128
98	2.8	52.0	2.4	51.4	36	2.5	51.4	72	2.1	49.3	129
102	2.8	51.1	2.3	50.7	34	2.6	50.7	77	2.0	48.9	124
Mean for weeks											
1-13	3.0	22.5	2.9	22.7	98	2.8	22.6	190	2.4	22.1	335
14-52	2.6	46.0	2.4	46.9	41	2.4	45.8	81	2.3	43.2	168
53-102	2.7	55.0	2.4	54.9	33	2.4	54.6	67	2.1	52.8	121

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[a] Grams of water consumed per animal per day

[b] Milligrams of sodium nitrite consumed per kilogram body weight per day

APPENDIX L
INGREDIENTS, NUTRIENT COMPOSITION, AND CONTAMINANT LEVELS IN NTP-2000 RAT AND MOUSE RATION

TABLE L1 Ingredients of NTP-2000 Rat and Mouse Ration 268
TABLE L2 Vitamins and Minerals in NTP-2000 Rat and Mouse Ration 268
TABLE L3 Nutrient Composition of NTP-2000 Rat and Mouse Ration 269
TABLE L4 Contaminant Levels in NTP-2000 Rat and Mouse Ration 270

TABLE L1
Ingredients of NTP-2000 Rat and Mouse Ration

Ingredients	Percent by Weight
Ground hard winter wheat	22.26
Ground #2 yellow shelled corn	22.18
Wheat middlings	15.0
Oat hulls	8.5
Alfalfa meal (dehydrated, 17% protein)	7.5
Purified cellulose	5.5
Soybean meal (49% protein)	5.0
Fish meal (60% protein)	4.0
Corn oil (without preservatives)	3.0
Soy oil (without preservatives)	3.0
Dried brewer's yeast	1.0
Calcium carbonate (USP)	0.9
Vitamin premix[a]	0.5
Mineral premix[b]	0.5
Calcium phosphate, dibasic (USP)	0.4
Sodium chloride	0.3
Choline chloride (70% choline)	0.26
Methionine	0.2

[a] Wheat middlings as carrier

[b] Calcium carbonate as carrier

TABLE L2
Vitamins and Minerals in NTP-2000 Rat and Mouse Ration[a]

	Amount	Source
Vitamins		
A	4,000 IU	Stabilized vitamin A palmitate or acetate
D	1,000 IU	D-activated animal sterol
K	1.0 mg	Menadione sodium bisulfite complex
α-Tocopheryl acetate	100 IU	
Niacin	23 mg	
Folic acid	1.1 mg	
d-Pantothenic acid	10 mg	*d*-Calcium pantothenate
Riboflavin	3.3 mg	
Thiamine	4 mg	Thiamine mononitrate
B_{12}	52 µg	
Pyridoxine	6.3 mg	Pyridoxine hydrochloride
Biotin	0.2 mg	*d*-Biotin
Minerals		
Magnesium	514 mg	Magnesium oxide
Iron	35 mg	Iron sulfate
Zinc	12 mg	Zinc oxide
Manganese	10 mg	Manganese oxide
Copper	2.0 mg	Copper sulfate
Iodine	0.2 mg	Calcium iodate
Chromium	0.2 mg	Chromium acetate

[a] Per kg of finished product

TABLE L3
Nutrient Composition of NTP-2000 Rat and Mouse Ration

Nutrient	Mean ± Standard Deviation	Range	Number of Samples
Protein (% by weight)	13.8 ± 0.72	12.7 – 16.2	23
Crude fat (% by weight)	8.1 ± 0.33	7.5 – 8.7	23
Crude fiber (% by weight)	9.5 ± 0.59	7.8 – 10.3	23
Ash (% by weight)	5.0 ± 0.16	4.8 – 5.4	23
Amino Acids (% of total diet)			
Arginine	0.732 ± 0.050	0.670 – 0.800	6
Cystine	0.220 ± 0.011	0.210 – 0.240	6
Glycine	0.683 ± 0.048	0.620 – 0.740	6
Histidine	0.333 ± 0.020	0.310 – 0.350	6
Isoleucine	0.522 ± 0.054	0.430 – 0.590	6
Leucine	1.065 ± 0.070	0.960 – 1.130	6
Lysine	0.705 ± 0.066	0.620 – 0.790	6
Methionine	0.402 ± 0.042	0.350 – 0.460	6
Phenylalanine	0.600 ± 0.042	0.540 – 0.640	6
Threonine	0.512 ± 0.056	0.430 – 0.590	6
Tryptophan	0.125 ± 0.015	0.110 – 0.150	6
Tyrosine	0.410 ± 0.037	0.360 – 0.460	6
Valine	0.628 ± 0.052	0.550 – 0.690	6
Essential Fatty Acids (% of total diet)			
Linoleic	3.98 ± 0.325	3.59 – 4.54	6
Linolenic	0.30 ± 0.048	0.21 – 0.35	6
Vitamins			
Vitamin A (IU/kg)	5,018 ± 1,421	2,780 – 8,140	23
Vitamin D (IU/kg)	1,000[a]		
α-Tocopherol (ppm)	77.2 ± 10.94	62.2 – 87.1	6
Thiamine (ppm)[b]	9.4 ± 2.19	6.0 – 15.0	23
Riboflavin (ppm)	5.6 ± 1.24	4.20 – 7.70	6
Niacin (ppm)	73.1 ± 4.13	66.4 – 78.8	6
Pantothenic acid (ppm)	24.2 ± 2.92	21.4 – 29.1	6
Pyridoxine (ppm)[b]	9.37 ± 2.50	6.7 – 12.4	6
Folic acid (ppm)	1.70 ± 0.43	1.26 – 2.32	6
Biotin (ppm)	0.349 ± 0.18	0.225 – 0.704	6
Vitamin B_{12} (ppb)	83.4 ± 67.1	30.0 – 174.0	6
Choline (ppm)	3,082 ± 232	2,700 – 3,400	6
Minerals			
Calcium (%)	0.958 ± 0.051	0.858 – 1.050	23
Phosphorus (%)	0.577 ± 0.022	0.548 – 0.640	23
Potassium (%)	6.660 ± 0.026	0.627 – 0.691	6
Chloride (%)	0.356 ± 0.031	0.300 – 0.392	6
Sodium (%)	0.193 ± 0.020	0.160 – 0.212	6
Magnesium (%)	0.197 ± 0.010	0.185 – 0.213	6
Sulfur (%)	0.182 ± 0.023	0.153 – 0.209	6
Iron (ppm)	158 ± 15.2	135 – 173	6
Manganese (ppm)	51.8 ± 4.05	46.2 – 56.0	6
Zinc (ppm)	53.2 ± 5.68	45.0 – 61.1	6
Copper (ppm)	6.49 ± 0.786	5.38 – 7.59	6
Iodine (ppm)	0.487 ± 0.204	0.233 – 0.843	6
Chromium (ppm)	0.763 ± 0.620	0.330 – 2.000	6
Cobalt (ppm)	0.53 ± 0.720	0.20 – 2.0	6

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[a] From formulation

[b] As hydrochloride

TABLE L4
Contaminant Levels in NTP-2000 Rat and Mouse Ration[a]

	Mean ± Standard Deviation[b]	Range	Number of Samples
Contaminants			
Arsenic (ppm)	0.26 ± 0.11	0.10 – 0.50	23
Cadmium (ppm)	0.05 ± 0.01	0.04 – 0.10	23
Lead (ppm)	0.14 ± 0.10	0.06 – 0.4	23
Mercury (ppm)	<0.02		23
Selenium (ppm)	0.18 ± 0.68	0.10 – 0.40	23
Aflatoxins (ppb)	<5.00		23
Nitrate nitrogen (ppm)[c]	13.2 ± 5.79	4.80 – 31.90	23
Nitrite nitrogen (ppm)[c]	0.85 ± 0.672	0.30 – 3.20	23
BHA (ppm)[d]	1.3 ± 1.04	0.01 – 5.00	23
BHT (ppm)[d]	1.2 ± 0.96	0.01 – 5.00	23
Aerobic plate count (CFU/g)[e]	156,770 ± 306,635	7,700 – 1,000,000	10
Coliform (MPN/g)[e]	17 ± 20	3 – 70	10
Escherichia coli (MPN/g)	<10		23
Salmonella (MPN/g)	Negative		23
Total nitrosoamines (ppb)[f]	6.5 ± 4.10	2.1 – 20.9	23
N-Nitrosodimethylamine (ppb)[f]	3.5 ± 2.50	1.0 – 9.4	23
N-Nitrosopyrrolidine (ppb)[f]	2.9 ± 2.70	1.0 – 14.5	23
Pesticides (ppm)			
α-BHC	<0.01		23
β-BHC	<0.02		23
γ-BHC	<0.01		23
δ-BHC	<0.01		23
Heptachlor	<0.01		23
Aldrin	<0.01		23
Heptachlor epoxide	<0.01		23
DDE	<0.01		23
DDD	<0.01		23
DDT	<0.01		23
HCB	<0.01		23
Mirex	<0.01		23
Methoxychlor	<0.05		23
Dieldrin	<0.01		23
Endrin	<0.01		23
Telodrin	<0.01		23
Chlordane	<0.05		23
Toxaphene	<0.10		23
Estimated PCBs	<0.20		23
Ronnel	<0.01		23
Ethion	<0.02		23
Trithion	<0.05		23
Diazinon	<0.10		23
Methyl chlorpyrifos	0.053 ± 0.051	0.010 – 0.160	22
Methyl parathion	<0.02		23
Ethyl parathion	<0.02		23
Malathion	0.138 ± 0.195	0.020 – 0.830	23
Endosulfan I	<0.01		23
Endosulfan II	<0.01		23
Endosulfan sulfate	<0.03		23

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[a] CFU=colony-forming units; MPN=most probable number; BHC=hexachlorocyclohexane or benzene hexachloride

[b] For values less than the limit of detection, the detection limit is given as the mean.

[c] Sources of contamination: alfalfa, grains, and fish meal

[d] Sources of contamination: soy oil and fish meal

[e] Nonirradiated samples; microbial counts for irradiated samples were below the detection limit.

[f] All values were corrected for percent recovery.

APPENDIX M
SENTINEL ANIMAL PROGRAM

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SENTINEL ANIMAL PROGRAM

METHODS

Rodents used in the Carcinogenesis Program of the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicologic evaluation of chemical compounds. Under this program, the disease state of the rodents is monitored via serology on sera from extra (sentinel) animals in the study rooms. These animals and the study animals are subject to identical environmental conditions. The sentinel animals come from the same production source and weanling groups as the animals used for the studies of chemical compounds.

Serum samples were collected from randomly selected rats and mice during the 14-week and 2-year studies. Blood from each animal was collected and allowed to clot, and the serum was separated. The samples were processed appropriately and sent to Microbiological Associates, Inc. (Bethesda, MD), for determination of antibody titers. The laboratory serology methods and viral agents for which testing was performed are tabulated below; the times at which blood was collected during the studies are also listed.

Method and Test	Time of Analysis
RATS	
14-Week Study	
ELISA	
PVM (pneumonia virus of mice)	Study termination
RCV/SDA (rat coronavirus/sialodacryoadenitis virus)	Study termination
Sendai	Study termination
Hemagglutination Inhibition	
H-1 (Toolan's H-1 virus)	Study termination
KRV (Kilham rat virus)	Study termination
2-Year Study	
ELISA	
Mycoplasma arthritidis	Study termination
Mycoplasma pulmonis	Study termination
PVM	1, 6, 12, and 18 months, study termination
RCV/SDA	1, 6, 12, and 18 months, study termination
Sendai	1, 6, 12, and 18 months, study termination
Immunofluorescence Assay	
M. arthritidis	Study termination
Hemagglutination Inhibition	
H-1	1, 6, 12, and 18 months, study termination
KRV	1, 6, 12, and 18 months, study termination

MICE

14-Week Study

ELISA

Ectromelia virus	Study termination
GDVII (mouse encephalomyelitis virus)	Study termination
LCM (lymphocytic choriomeningitis virus)	Study termination
Mouse adenoma virus	Study termination
MHV (mouse hepatitis virus)	Study termination
PVM	Study termination
Reovirus 3	Study termination
Sendai	Study termination

Immunofluorescence Assay

EDIM (epizootic diarrhea of infant mice)	Study termination
MVM (minute virus of mice)	Study termination

Hemagglutination Inhibition

K (papovavirus)	Study termination
Polyoma virus	Study termination

2-Year Study

ELISA

Ectromelia virus	1, 6, 12, and 18 months, study termination
EDIM	1, 6, 12, and 18 months, study termination
GDVII	1, 6, 12, and 18 months, study termination
LCM	1, 6, 12, and 18 months, study termination
Mouse adenoma virus-FL	1, 6, 12, and 18 months, study termination
MHV	1, 6, 12, and 18 months, study termination
M. arthritidis	Study termination
M. pulmonis	Study termination
PVM	1, 6, 12, and 18 months, study termination
Reovirus 3	1, 6, 12, and 18 months, study termination
Sendai	1, 6, 12, and 18 months, study termination

Immunofluorescence Assay

Ectromelia virus	Study termination
EDIM	6 months
GDVII	Study termination
LCM	Study termination
Mouse adenoma virus-FL	Study termination
MCMV (mouse cytomegalovirus)	Study termination
MHV	6 months, study termination
PVM	1 month, study termination

Hemagglutination Inhibition

K	1, 6, 12, and 18 months, study termination
MVM	1, 6, 12, and 18 months, study termination
Polyoma virus	1, 6, 12, and 18 months, study termination

RESULTS

All test results were negative.

Acta Physiol Scand 2001, **171**, 169–176

Effect of creatine supplementation on creatine and glycogen content in rat skeletal muscle

B. OP 'T EIJNDE, E. A. RICHTER, J.-C. HENQUIN, B. KIENS and P. HESPEL

Faculty of Physical Education and Physiotherapy, Department of Kinesiology, Katholieke Universiteit Leuven, and Endocrinology and Metabolism Unit, Université Catholique de Louvain, Belgium and The Copenhagen Muscle Research Centre, August Krogh Institute, University of Copenhagen, Denmark

ABSTRACT

The effects of high dose creatine feeding (5 g kg^{-1} BW day^{-1}, 5 days) on creatine content, glucose transport, and glycogen accumulation in white gastrocnemius, red gastrocnemius and soleus muscles of the rat was investigated. Isolated rat hindquarters of creatine fed and control rats were perfused with a standard medium containing either insulin alone (0, 100 or 20 000 $\mu U\ mL^{-1}$) or in combination with creatine (2 or 10 mmol L^{-1}). Furthermore, plasma insulin concentration was measured in normal rats during creatine feeding, as well as in anaesthetized rats during intravenous creatine infusion. Five days of creatine feeding increased ($P < 0.05$) total creatine content in soleus (+20%) but not in red gastrocnemius (+15%, n.s.) and white gastrocnemius (+10%, n.s.). In parallel, glycogen content was markedly elevated ($P < 0.05$) in soleus (+40%), less ($P < 0.05$) in red gastrocnemius (+15%), and not in white gastrocnemius (+10%, n.s.). Glucose transport rate, muscle GLUT-4 content, glycogen synthase activity in perfused muscles and glycogen synthesis rate were not significantly altered by creatine feeding in either muscle type. Furthermore, high dose creatine feeding raised ($P < 0.05$) plasma creatine concentration fivefold but did not alter circulating insulin level. It is concluded that short-term high dose creatine feeding enhances creatine disposal and glycogen storage in rat skeletal muscle. However, the creatine and glycogen response to creatine supplementation is markedly greater in oxidative than in glycolytic muscles.

Keywords glucose uptake, insulin, muscle, phosphocreatine.

Received 19 May 2000, accepted 12 October 2000

The sensitivity of skeletal muscle glucose transport to stimulation by insulin varies considerably among individuals. Abnormally low insulin-mediated glucose transport, probably combined with inadequate compensatory hyperinsulinaemia, is the primary pathogenic mechanism underlying reduced glucose incorporation into muscle glycogen in non-insulin-dependent diabetes mellitus (NIDDM) (Shepherd & Kahn 1999). Therefore, any procedure capable of enhancing insulin stimulation of skeletal muscle glucose disposal may have significant therapeutic potential in the treatment of NIDDM.

After Harris *et al.* (1992) demonstrated for the first time the efficacy of oral creatine intake to increase skeletal muscle creatine content in humans, interest in the effects of oral creatine supplementation on skeletal muscle contractile performance and metabolism has rapidly increased. Today it is well established that oral creatine 'loading', notably the intake of ~20 g of creatine monohydrate per day for 4–5 days, may raise muscle phosphocreatine level by about 5–10% (Harris *et al.* 1992, Vandenberghe *et al.* 1997) particularly in individuals having low initial muscle creatine content (Harris *et al.* 1992, Greenhaff *et al.* 1994). This higher intracellular creatine availability can increase muscle force and power output during high-intensity exercise (Greenhaff *et al.* 1994, Vandenberghe *et al.* 1997, American College of Sports Medicine, 2000). Moreover, we have recently demonstrated prolonged creatine intake to stimulate muscle hypertrophy during high-resistance training (Vandenberghe *et al.* 1997).

The beneficial effects of creatine supplementation on muscle performance capacity in athletic populations

Correspondence: Peter Hespel, Faculty of Physical Education and Physiotherapy, Exercise Physiology Laboratory, Tervuursevest 101, B-3001 Leuven, Belgium.

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have prompted interest in the clinical potential of creatine intake, particularly with regard to the treatment of neuromuscular diseases. Experiments in animal models of neurodegenerative disease, including Parkinson's disease (Matthews *et al.* 1999), Huntington's disease (Matthews *et al.* 1998) and amyotrophic lateral sclerosis (ALS) (Klivenyi *et al.* 1999) have shown dietary creatine supplementation to inhibit neuronal necrosis. Furthermore, recent studies have shown that supplementary oral creatine is a cost-effective strategy to treat various types of mitochondrial cytopathies (Tarnopolsky & Martin 1999) and to enhance the recovery of muscle mass and functional capacity following disuse atrophy (Hespel *et al.* unpublished observations). Thus, oral creatine supplementation may conceivably rapidly develop as a standard therapeutic strategy in the above clinical conditions.

Interestingly, some recent studies also have indicated the potential of oral creatine supplementation to promote muscle glucose uptake. In this respect, Greenhaff and colleagues reported that carbohydrate intake in conjunction with creatine ingestion, resulted in greater muscle glycogen accumulation than carbohydrate intake alone (Robinson *et al.* 1999). In addition, they found a close correlation between the extent of muscle glycogen and creatine accumulation (Green *et al.* 1996). Furthermore, preliminary observations in both healthy subjects (Op 't Eijnde *et al.* unpublished observations) and diabetic patients (Rocic *et al.* 1995) indicate that creatine supplementation might facilitate peripheral glucose disposal.

The physiological mechanisms underlying this beneficial effect of creatine supplementation on muscle glycogen accumulation is unknown at present. However, given the pivotal role of insulin in the stimulation of muscle glucose uptake, facilitated insulin secretion, alone or in combination with enhanced muscle insulin sensitivity, are likely mechanisms to promote glucose uptake and glycogen synthesis in skeletal muscle during creatine supplementation. In this respect, *in vitro* studies using the isolated and perfused rat pancreas (Alsever *et al.* 1970), isolated mouse islets (Marco *et al.* 1976) or incubated INS-1 cells (Gempel *et al.* 1996) have already documented the insulin-releasing effects of guanidino-acetate compounds, including creatine.

We hypothesized that stimulation of insulin secretion, possibly in conjunction with enhanced sensitivity of skeletal muscle tissue to insulin, is responsible for increasing muscle glycogen storage during creatine supplementation. Therefore, the impact of both acute and short-term creatine supplementation on plasma insulin concentration on the one hand, and on sensitivity of muscle glucose uptake to stimulation by insulin on the other hand, was evaluated in rats.

METHODS

Animals

Male Wistar rats weighing 200–230 g were used. They were purchased from the laboratory animal-breeding centre at the Catholic University of Leuven. Rats were maintained on a constant light-to-dark cycle (12 : 12) and 1 week before the start of the experiments were accustomed to powder feeding. Thereafter, during the 5 days preceding the experiments, rats were housed in individual cages and received 20 g day^{-1} of normal powdered rat chow alone (C: control rats) or containing 5% of creatine monohydrate (CR: creatine-loaded rats). All rats consumed the entire 20 g daily chow allowance throughout the 5-day supplementation period. The experimental procedures were approved by the local animal welfare committee.

Hindlimb perfusion experiments

Perfusion protocol. Rats were anaesthetized by an intra-peritoneal injection of pentobarbital sodium (5 mg 100 g^{-1} BW) and prepared surgically for hindquarter perfusion as previously described (Ruderman *et al.* 1971). Before insertion of the perfusion catheters, the rat was heparinized with 250 IU of heparin in the inferior vena cava. The rat was then immediately killed by an intra-cardial injection of pentobarbital sodium before being placed in the perfusion cabinet. The initial perfusate (150 mL) consisted of Krebs–Henseleit solution, 1–3-day-old washed bovine erythrocytes at a haematocrit of 30%, 5% bovine serum albumin (Cohn fraction V; Sigma, St Louis, MO, USA), 6 mmol L^{-1} glucose, 0.15 mmol L^{-1} pyruvate, and 0.1–0.3 mmol L^{-1} lactate originating from the erythrocytes. During the perfusion experiments, the arterial perfusate was continuously gassed with a mixture of 62% N_2, 35% O_2 and 3% CO_2, yielding arterial pH, PO_2 and PCO_2 values of 7.30–7.45, 100–250 mmHg and 30–45 mmHg, respectively. The arterial glucose concentration during the perfusion was clamped at a concentration of 6 mmol L^{-1} by continuous infusion of a 40% glucose solution into the perfusate reservoir, using a microinfusion pump (Harvard model 11, South Natick, MA, USA). The speed of infusion was adjusted to the predicted rate of glucose uptake by the hindquarter. The first 25 mL of perfusate that passed through the hindquarter were discarded, at which point the perfusate was recirculated at a flow of 12.5 mL min^{-1} for 15 min allowing perfusion pressure and the metabolic state of the hindquarter to stabilize. At the end of this equilibration period perfusate flow was maintained at 12.5 mL min^{-1}. A 10-min experimental period was started at the end of which arterial and venous perfusate samples were taken. Perfusate flow rate was measured immediately preceding the sampling of perfusate using time collections of the venous effluent from the hindlimb. Perfusion pressures were read from a manometer connected to an arterial tubing of the perfusion apparatus. At the end of the experimental period muscle tissue was also sampled. The medial superficial part of the gastrocnemius, consisting mainly of fast-glycolytic fibres, the soleus muscle, consisting mainly of slow-oxidative fibres, and a deep part of the medial head of the gastrocnemius, consisting mainly of fast-oxidative fibres, were cut out from the left leg, trimmed of connective tissue and visible blood and freeze clamped with aluminium clamps cooled in liquid N_2.

Experimental conditions. All hindquarter perfusions were performed according to the above-described standard procedures. However, different experimental conditions were created by varying insulin and creatine concentrations in the perfusate mixture. Thus, perfusions were carried out with either 0, 100 or 20 000 μU insulin mL^{-1} of cell free perfusate added to the perfusion mix (human monocomponent insulin, Actrapid®, Novo Nordisk, Bagsværd, Denmark). Furthermore, a separate group of control rats was perfused at 100 μU mL^{-1} insulin concentration, together with either 2 or 10 mmol L^{-1} plasma creatine concentrations (Sigma Biochemicals, St Louis, MO, USA). To compensate for increased perfusate osmolality caused by the addition of creatine in these experiments, a similar concentration of mannitol (Sigma Biochemicals) was added to corresponding control perfusates.

Glucose transport measurements. Muscle membrane glucose transport was measured in a separate experiment in creatine loaded and control rats perfused at 100 μU mL^{-1} insulin with no creatine added to the perfusate. 2-Deoxy-D-2,6-[^{3}H] glucose (Amersham, specific activity 51.0 Ci $mmol^{-1}$) and D-1-[^{14}C] mannitol (Amersham, specific activity 57.0 mCi $mmol^{-1}$) were added simultaneously to the glucose-free perfusate, yielding activities of ~0.050 μCi mL^{-1} and ~0.075 μCi mL^{-1} for ^{3}H and ^{14}C, respectively (Wojtaszewski *et al.* 1998). The isotopes reached the hindlimbs exactly at the end of the equilibration period. Simultaneously, a stock solution of cold 2-deoxy-D-glucose and D-mannitol was added to the initial perfusate yielding 6 and 1 mmol L^{-1} concentrations of cell-free perfusate, respectively. When the isotopes and stock solution were added to the initial perfusate, recirculation was stopped and one-way perfusion was started to secure a constant specific activity for 2-deoxy-D-glucose in the arterial perfusate during the 10-min period of glucose transport measurement following the equilibration period. At the end of a 10-min perfusion period, muscle samples were taken as described above.

Effect of creatine on plasma insulin

In a separate series of experiments circulating plasma insulin and blood glucose concentrations were measured in rats during high dose creatine feeding. Rats were housed in individual cages and were pre-conditioned for 1 week to time-limited feeding and experimental manipulation for a period of 4 h day^{-1}. After an overnight fast, rats received 5 g of powdered rat chow at 9.00 a.m. Additional 5 g dosages were given 1, 2 and 3 h later. Residual food was removed at 1.00 p.m; thereafter rats remained fasting until the next morning. Thus, total food intake amounted to 16–20 g day^{-1} during the 7 days of pre-conditioning. On the experimental day at 9.00 a.m., a 200-μL blood sample was collected from the heated tail and placed into heparinized glass capillaries by cutting ~1 mm of the end of the heated tail under light ether anaesthesia. Immediately thereafter rats received 5 g of powdered rat chow containing 5% creatine vs. pure rat chow in controls. Exactly 1 h later a second blood sample (600–800 μL) was taken. Finally, the residual food was weighed to quantify the food intake during the 1-h experimental re-feeding period. Blood samples were immediately assayed for glucose concentration. Plasma was then separated by high-speed centrifugation and stored at −80 °C until analysed for plasma insulin and creatine concentrations.

Biochemical analyses and calculations

Perfusate and rat blood samples were analysed for glucose concentration in at least duplicate by a glucose-oxidase method, using an automated glucose analyser (Yellow Springs Instruments, model 2300 STAT, OH, USA). Rate of glucose uptake by the rat hindquarter was calculated by multiplying the arteriovenous concentration differences measured by the corresponding flow rate and were expressed per gram of perfused muscle per hour. Perfused muscle mass was considered to be 16.6% of body weight (Ruderman *et al.* 1971). Plasma samples were stored at −80 °C until assayed. Insulin concentration was determined by a double-antibody radio-immunoassay with rat insulin as the standard (Novo Research Institute, Bagsværd, Denmark). Plasma creatine concentration was measured using a standard enzymatic fluorometric assay (Bergmeyer 1985).

Muscle samples were stored at −80 °C until analysed. Total muscle water was measured by weighing the samples before and after freeze-drying. Thereafter, muscle glycogen was determined by a hexokinase method after acid hydrolysis (Lowry & Passoneau

1972). Muscle ATP, phosphocreatine and free creatine content was analysed from perchloric acid precipitated extractions of the freeze-dried muscle samples using standard fluorometric assays (Bergmeyer 1985). Muscle total creatine content was calculated as the sum of free creatine and phosphocreatine. All data are expressed in mmol kg^{-1} DW.

Glycogen synthase activity was determined using a modification of the filter paper method (Thomas *et al.* 1968). Briefly, glycogen synthase fractional activity was assayed at 0.17 mmol L^{-1} glucose-6-phosphate in the assay mixture and expressed as a percentage of the total synthase activity measured in the presence of 8 mmol L^{-1} glucose-6-phosphate.

Glucose transport was measured by deproteinizing 100 μL of the initial perfusate plasma taken 5 min after the end of the equilibration period in 1 mL 0.6 mol L^{-1} perchloric acid. The corresponding muscle samples, which were stored at –80 °C until analysed, were first homogenized and deproteinized in 1 mL of 0.6 mol L^{-1} perchloric acid. Both plasma and muscle solutions were centrifuged for 10 min at 3000 *g*, thereafter 400 μL of supernatant was mixed in duplicate with 3 mL of LSC cocktail (Ultima Gold, Packard, USA). Isotopes were then counted by an LS counter (1600 TR Liquid Scintillator Analyser, Packard, USA).

Muscle total Glut-4 content was determined in total crude muscle membranes (TCM) which were prepared as described previously (Ploug *et al.* 1993). Samples were loaded on a discontinuous gradient (8–18%) SDS gel, electrophoresed on a horizontal Multiphor system (Pharmacia, Uppsala, Sweden), and then transferred to an Immobilon membrane (Millipore, Bedford, MA, USA) by electrotransfer. We used a mouse monoclonal primary antibody directed against a synthetic peptide corresponding to the ^{13}C-terminal amino acids of GLUT-4. The second antibody was horseradish peroxidase-labelled goat antimouse antibody. Antibody–antigen complexes were visualized within the linear response of the enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA) on hyperfilm. To quantify the signal densitometric scanning was performed (CREAM software; Kem-En-Tec Software Systems, Copenhagen, Denmark).

Statistical analyses

Statistical evaluation of the data was performed by unpaired *t*-test or repeated measures of two-way analysis of variance where appropriate, using Statistica software (Statsoft, Tulsa, OK, USA). Data are presented as mean ± SEM. A probability level of $P < 0.05$ was chosen as the threshold for acceptance of statistical significance.

RESULTS

Muscle metabolites

Compared with C, *in vivo* creatine loading increased ($P < 0.05$) creatine (~30%) and phosphocreatine (10–15%) content in SOL (Table 1). It also increased total creatine and glycogen content ($P < 0.05$) by ~20% and ~40% in SOL and by 11 and 15% in RG, respectively (Table 1). In contrast, creatine loading did not significantly alter creatine and phosphocreatine in RG and WG, or glycogen in WG. Muscle ATP and H_2O content (Table 1) and glycogen synthase fractional velocity (Table 2) were similar in CR and C in all muscle types.

Muscle glucose uptake and transport

The effect of creatine supplementation on basal and insulin-stimulated muscle glucose uptake was evaluated in perfused rat hindquarters. As shown in Table 3, compared with values in the absence of insulin, hindlimb glucose uptake rate was increased by a factor ~3 and ~6 by the addition of a submaximal (100 μU mL^{-1}) and a supramaximal (20 000 μU mL^{-1})

Table 1 Effect of creatine supplementation on muscle glycogen, creatine, phosphocreatine, total creatine, ATP and H_2O content

	White gastrocnemius	Red gastrocnemius	Soleus
Glycogen (*n* = 13)			
Control	194 ± 10	218 ± 13	127 ± 7
Creatine loaded	213 ± 9	253 ± 12*	178 ± 11*
Creatine (*n* = 13)			
Control	54 ± 2	52 ± 4	35 ± 2
Creatine loaded	59 ± 4	59 ± 4	45 ± 3*
Phosphocreatine (*n* = 13)			
Control	76 ± 3	64 ± 3	47 ± 2
Creatine loaded	81 ± 3	70 ± 3	54 ± 2*
Total creatine (*n* = 13)			
Control	130 ± 3	116 ± 4	82 ± 2
Creatine loaded	140 ± 5	129 ± 5*	99 ± 4*
ATP (*n* = 13)			
Control	28.4 ± 0.9	23.7 ± 1.3	17.7 ± 0.6
Creatine loaded	25.4 ± 1.0*	25.8 ± 0.9	18.2 ± 0.6
H_2O (*n* = 13)			
Control	77.0 ± 0.2	76.3 ± 0.4	78.3 ± 0.7
Creatine loaded	76.9 ± 0.8	75.8 ± 0.3	77.1 ± 0.1

Values are mean ± SEM and are expressed in mmol kg^{-1} DW (dry muscle weight). Muscle H_2O content is expressed as a percentage of the total muscle sample weight. Muscle samples were taken from rat hindlimbs perfused at 100 μU mL^{-1} insulin concentration following either 5 days of creatine administration (20 g powdered rat chow per day containing 5% of creatine monohydrate) or matched control feeding. See METHODS for further details. *$P < 0.05$ compared with corresponding control value.

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Table 2 Effect of creatine supplementation on muscle GLUT-4 content, glucose transport, glycogen synthase activity and glycogen synthesis rate

	White gastrocnemius	Red gastrocnemius	Soleus
GLUT-4 (n = 10)			
Control	29 ± 4	55 ± 5	80 ± 5
Creatine loaded	35 ± 5	54 ± 6	71 ± 6
Glucose transport (n = 7)			
Control	3.8 ± 0.8	18.0 ± 1.8	10.4 ± 1.7
Creatine loaded	3.0 ± 0.7	15.5 ± 2.1	9.7 ± 1.3
Glycogen synthase activity (n = 8)			
Total activity			
Control	0.53 ± 0.04	1.13 ± 0.04	1.08 ± 0.09
Creatine loaded	0.59 ± 0.04	0.95 ± 0.16	1.12 ± 0.06
Fractional velocity			
Control	41.6 ± 2.5	35.3 ± 2.4	37.8 ± 2.9
Creatine loaded	42.6 ± 2.6	33.8 ± 4.2	33.9 ± 3.5

Values are mean ± SEM. GLUT-4 is expressed as a percentage of a rat heart standard mg^{-1} protein. Glucose transport is expressed in μmol g^{-1} h^{-1}. Glycogen synthase total activity is expressed in nmol min^{-1} mg^{-1} WW (wet muscle weight). Glycogen synthase fractional velocity (GS) is expressed as a percentage of the corresponding total glycogen synthase activity. Muscle samples were taken from rat hindlimbs perfused at 100 μU mL^{-1} insulin concentration following either 5 days of creatine administration (20 g powdered rat chow per day containing 5% of creatine monohydrate) or matched control feeding. Glycogen synthesis rate was measured as the incorporation of [^{14}C]-glucose label in muscle glycogen in hindlimbs perfused at 50 μU mL^{-1} insulin at either 0 (control) or 5 (creatine) mmol L^{-1} perfusate creatine concentration. See METHODS for further details. *$P < 0.05$ compared with corresponding control value.

Table 3 Effect of increased insulin and creatine concentrations on muscle glucose uptake in creatine supplemented rats

Perfusate concentrations		Glucose uptake (μmol g^{-1} h^{-1})	
Insulin (μU mL^{-1})	Creatine (mmol L^{-1})	Control	Creatine loaded
0	0 (n = 8)	3.0 ± 0.6	2.6 ± 0.4
100	0 (n = 7)	9.2 ± 0.9	9.5 ± 0.9
	2 (n = 10	8.1 ± 1.0	9.1 ± 0.6
	10 (n = 6)	10.5 ± 0.9	9.4 ± 0.8
20 000	0 (n = 8)	20.6 ± 2.0	19.3 ± 1.2

Values are mean ± SEM. Glucose uptake was measured in hindlimbs perfused at either 0, 100 or 20 000 μU mL^{-1} plasma insulin and/or either 0, 2 and 10 mmol L^{-1} perfusate plasma creatine concentrations, following either 5 days of creatine administration (20 g of powdered rat chow per day containing 5% of creatine monohydrate) or matched control feeding. Glucose uptakes were calculated from the arteriovenous glucose concentration differences times the flow rate. See METHODS for further details.

Table 4 Effect of creatine feeding on plasma creatine, plasma insulin and blood glucose in rats

	Before feeding	1 h after feeding
Plasma creatine (n = 7)		
Control	0.12 ± 0.02	0.10 ± 0.02
Creatine	0.16 ± 0.02	0.61 ± 0.08*
Plasma insulin (n = 14)		
Control	27.3 ± 5.4	69.4 ± 9.5
Creatine	22.5 ± 4.4	56.5 ± 9.8
Blood glucose (n = 14)		
Control	3.79 ± 0.09	4.45 ± 0.09
Creatine	3.64 ± 0.05	4.67 ± 0.11

Values are mean ± SEM. Plasma creatine, plasma insulin and blood glucose are expressed in mmol L^{-1}, μU mL^{-1} and mmol L^{-1}, respectively. Blood samples were taken from the heated tail immediately before and 1 h after the administration of 5 g of powdered rat chow, containing 5% of creatine monohydrate, or matched control feeding. See METHODS for further details. *$P < 0.05$ compared with corresponding control value.

insulin concentration to the perfusate mixture, respectively. However, glucose uptake rate was not altered by 5 days of prior creatine loading (Table 3). Accordingly, muscle glucose transport, measured as the rate of intracellular accumulation of ^{3}H labelled deoxy-glucose at 100 μU mL^{-1} plasma insulin concentration, and muscle total GLUT-4 content, were similar in CR and C in either muscle type (Table 2). In fact we also measured glucose uptake in rat hindquarters perfused after either 1 or 3 days of creatine supplementation (data not shown). Values were similar to values measured after 5 days of creatine supplementation (Table 2), and were also independent of creatine supplementation. In C and CR muscles extracellular space (estimated by the accumulation of ^{14}C-mannitol in muscle tissue), was on average 9.5 ± 0.3, 12.9 ± 0.6 and 14.6 ± 0.5 mL 100 g^{-1} muscle in WG, RG and SOL, respectively.

To explore the possibility of a direct effect of extracellular creatine on muscle glucose uptake, hindlimbs were perfused at 100 μU mL^{-1} insulin with either 0, 2 or 10 mmol L^{-1} of creatine added to the perfusion mix. Table 3 shows that increased circulating creatine concentration, did not affect glucose uptake by the hindquarter neither in C nor in CR.

Plasma insulin

As shown in Table 4, addition of creatine to the rat chow during the 1 h re-feeding period, caused plasma creatine concentration to increase ($P < 0.05$) about fourfold, whereas plasma creatine remained stable in rats receiving control chow. Re-feeding markedly raised ($P < 0.05$) plasma insulin level in both groups of rats, but the magnitude of this increase was not affected by creatine supplementation. Accordingly, the increment of blood glucose concentration as well as the insulin : glucose concentration ratio during re-feeding, were similar in creatine-fed rats and controls.

DISCUSSION

It has been observed recently that oral creatine intake may markedly increase glycogen concentration in human skeletal muscle (Green *et al.* 1996, Robinson *et al.* 1999, Op 't Eijnde *et al.* unpublished observations). Here we show that this is also the case in rat skeletal muscle but that the effect is more prominent in oxidative than in glycolytic muscles. In addition, as the mechanisms behind the increase in muscle glycogen content with creatine supplementation are unknown, we explored the possible mechanisms. In spite of the markedly increased (40%) muscle glycogen content in slow oxidative muscle, creatine did, however, not measurably increase insulin sensitivity or plasma insulin concentration *in vivo*.

Skeletal muscle is the primary site of creatine storage in the human body. More than 70% of the creatine entering muscle cells is converted into phosphocreatine, the remainder being stored in the unphosphorylated form. Under normal conditions total muscle creatine pool is maintained within narrow limits. However, in men with high-dose oral creatine supplementation (4–5 days, ~20 g day^{-1}) it has recently been shown to have the potential to push muscle creatine balance towards net creatine accumulation (Harris *et al.* 1992, Vandenberghe *et al.* 1997). Moreover, recent findings have indicated that creatine intake in humans not only raises muscle creatine level, but also at the same time increases muscle glycogen content (Green *et al.* 1996, Robinson *et al.* 1999, Op 't Eijnde *et al.* unpublished observations). Accordingly, in the current experiment 5 days of creatine feeding in rats significantly increased the muscle intracellular creatine pool, particularly in slow-twitch muscle (see Table 1). Interestingly, creatine feeding also caused muscle glycogen store to be elevated to a much higher extent in slow-oxidative muscle fibres than in fast-glycolytic muscle. These findings are in keeping with recent data obtained in incubated rat muscles which showed that, because of the lower Km of the creatine transporter, lower extracellular creatine concentrations are needed to stimulate creatine uptake in *m. soleus* than in *m. extensor* digitorum longus (Willott *et al.* 1999). In apparent contrast with the observations in rat muscles, oral creatine loading in young male volunteers was recently reported to increase phosphocreatine content to the same degree (~15%) in quadriceps muscle type II and type I fibres (Casey *et al.* 1996). However, it should be noted that 'type II' fibres dissected from human muscle biopsies contain both 'glycolytic' type IIb and 'oxidative' type IIa fibres. In addition, compared with rats, the range of oxidative capacities as well as intracellular creatine content among muscle fibres (type I → IIb) is much narrower in humans than in rats (Schiaffino & Reggiani 1996).

Distinct muscle fibre types exhibit markedly different sensitivities to insulin. The stimulation of glucose transport is many-fold higher in slow-oxidative than in fast-glycolytic muscle fibres, the fast-oxidative fibres having an intermediate position (James *et al.* 1985). Because increased glucose transport is a likely pre-requisite for increased glycogen synthesis the greater glycogen accumulation occurring in oxidative muscle types upon creatine feeding suggests that insulin-stimulated glucose uptake might be enhanced after creatine supplementation. Furthermore, because both glucose transport and creatine transport (Haugland & Chang 1975, Odoom *et al.* 1996, Willott *et al.* 1999) into muscle cells is subject to stimulation by insulin, muscle creatine and glycogen trapping during creatine feeding may conceivably occur simultaneously, particularly in oxidative muscle fibres.

Unlike charged guanidine compounds such as arginine (Henquin 1990), creatine is not known as a potent acute insulin secretagogue. Still some *in vitro* studies have indicated that creatine may increase insulin secretion modestly in the perfused rat pancreas (Alsever *et al.* 1970), incubated mouse islets (Marco *et al.* 1976) and incubated insulinoma cells (Gempel *et al.* 1996). However, in the current study a large increase in plasma creatine concentration caused by creatine feeding or intravenous creatine injection, did not alter basal or glucose-stimulated plasma insulin level. In addition, oral intake of a 5 g creatine dosage in humans, which is known to raise plasma creatine about 10-fold (Harris *et al.* 1992), did not affect the circulating insulin concentration (Op 't Eijnde *et al.* unpublished observations). Thus, creatine clearly does not appear to acutely influence insulin secretion *in vivo*. Alternatively, the increased accumulation of muscle glycogen during creatine supplementation, might be caused by increased insulin action. Hence we evaluated the effect of creatine 'loading' on the sensitivity of muscle glucose uptake and glycogen synthesis to stimulation by insulin. Membrane glucose transport, rate of glycogen synthesis and glycogen synthase fractional activity in muscles perfused at a submaximal insulin concentration (100 $\mu\text{U mL}^{-1}$), were higher in oxidative muscle types than in fast-glycolytic muscles, but they were unaffected by either prior creatine loading or by acute exposure of muscles to a high extracellular creatine concentration. Furthermore, creatine supplementation also failed to increase maximal capacity for insulin-stimulated membrane glucose transport, as evidenced by unchanged muscle GLUT-4 content and correspondingly muscle glucose transport

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rate at a supramaximal insulin concentration. Thus, our current data indicate that creatine loading does not measurably enhance the sensitivity and the responsiveness of rat muscles to insulin. We cannot exclude the fact that the current glucose uptake and transport and glycogen synthesis measurements lacked sensitivity to detect the minor degree of glucose transport stimulation required to elevate muscle glycogen concentration by ~20–40% over a period of 5 days.

From the above findings it appears that enhanced muscle glycogen storage during creatine supplementation is not caused by facilitation of insulin-mediated muscle glucose transport and glycogen synthesis. Other putative mechanisms include cell swelling caused by elevated intracellular osmolality. It is well documented that cell swelling is a potent stimulus to glycogen synthesis in both muscle (Low *et al.* 1996) and liver (Baquet *et al.* 1990). Thus, increased myoplasmic osmolality, resulting from enhanced muscle creatine uptake, might contribute to the stimulation of glycogen synthesis during episodes of creatine supplementation. Such a contention is compatible with the greater accumulation of glycogen in those muscle types exhibiting the greater increase in total creatine concentration. Percentage muscle water was not increased following creatine supplementation (see Table 1). However, because muscle water content was measured relative to total muscle weight, it cannot be excluded that in the creatine-fed animals an increase in muscle weight caused by intracellular creatine and glycogen accumulation might have masked a 0.2–0.3% increase of muscle cell volume. Observations in isolated perfused rat liver have indicated that cell volume changes of such a magnitude might play a role in insulin action (Vom Dahl *et al.* 1991). Still, 5 mmol L^{-1} of creatine added to the perfusate did not increase glucose uptake or glycogen synthesis over 2 h perfusion which would be expected if enhanced glycogen synthesis was caused by increased intracellular volume because of creatine uptake. Other possible mechanisms for creatine effects include creatine stimulation of other hormones, e.g. IGF-1, which has insulin-like effects (Czech & Corvera 1999) and/or decreased secretion of insulin-antagonistic hormones. However, such putative effects are at present not backed up by data.

In conclusion, creatine loading increases glycogen concentration in rat skeletal muscles, and most prominently in slow-oxidative muscles. Concomitantly muscle glycogen content is enhanced. In the conditions of the current study, this 'glycogen-loading' action of creatine could not be explained by an increase in circulating insulin levels or to an increase in muscle insulin sensitivity. Given the pivotal role of impaired muscle glycogen storage in NIDDM it is warranted to further explore the physiological mechanisms underlying enhanced muscle glycogen storage during oral creatine supplementation.

The authors thank Betina Blomgreen, Irene B. Nielsen and Monique Ramaekers for providing skilled technical assistance. This study was supported by Grant G.0331.98 from the Fonds voor Wetenschappelijk Onderzoek Vlaanderen and Grant OT 94/31 and OT99/38 from the Onderzoeksraad K.U. Leuven and from the Danish National Research Foundation, Grant 504-14.

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C. Nucci et al. (Eds.)
Progress in Brain Research, Vol. 173
ISSN 0079-6123
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CHAPTER 24

Pathogenesis of ganglion "cell death" in glaucoma and neuroprotection: focus on ganglion cell axonal mitochondria

Neville N. Osborne*

Nuffield Laboratory of Ophthalmology, University of Oxford, Walton Street, Oxford OX2 6AW, UK

Abstract: Retinal ganglion cell axons within the globe are functionally specialized being richly provided with many mitochondria. The mitochondria produce the high energy requirement for nerve conduction in the unmyelinated part of the ganglion cell axons. We have proposed that in the initiation of glaucoma, an alteration in the quality of blood flow dynamics in the optic nerve head causes a compromise in the retinal ganglion cell axon energy requirement, rendering the ganglion cells susceptible to additional insults. One secondary insult might be light entering the eye to further affect ganglion cell axon mitochondrial function. Other insults to the ganglion cells might be substances (e.g., glutamate, nitric oxide, TNF-α) released from astrocytes. These effects ultimately cause ganglion cell death because of the inability of mitochondria to maintain normal function. We therefore suggest that ganglion cell apoptosis in glaucoma is both receptor and mitochondrial mediated. Agents targeted specifically at enhancing ganglion cell mitochondrial energy production should therefore be beneficial in a disease like glaucoma. Ganglion cell death in glaucoma might therefore, in principle, not be unlike the pathophysiology of numerous neurological disorders involving energy dysregulation and oxidative stress.

The trigger(s) for ganglion cell apoptosis in glaucoma is/are likely to be multifactorial, and the rationale for targeting impaired energy production as a possibility of improving a patient's quality of life is based on logic derived from laboratory studies where neuronal apoptosis is shown to occur via different mechanisms. Light-induced neuronal apoptosis is likely to be more relevant to ganglion cell death in glaucoma than, for example, neuronal apoptosis associated with Parkinson's disease. Logic suggests that enhancing mitochondrial function generally will slow down ganglion cell apoptosis and therefore benefit glaucoma patients. On the basis of our laboratory studies, we suggest that supplements such as creatine, α-lipoic acid, nicotinamide, and epigallocatechin gallate (EGCG), all of which counteract oxidative stress induced by light and other triggers, are worthy of consideration for the treatment of such patients as they can be taken orally to reach the retina without having significant side effects.

Keywords: pathogenesis of glaucoma; ganglion cells; mitochondria; apoptosis; light injury to mitochondria; neuroprotection; creatine; α-lipoic acid; nicotinamide; epigallocatechin gallate

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*Corresponding author. Tel.: +44 1865 248996;
Fax: +44 1865 794508; E-mail: neville.osborne@eye.ox.ac.uk

DOI: 10.1016/S0079-6123(08)01124-2

Introduction

Glaucoma, or glaucomatous optic neuropathy, is a chronic neurodegenerative disease characterized by a progressive loss of retinal ganglion cells (Quigley, 1999; Whitmore et al., 2005). The disease is associated with a specific remodeling of the optic nerve head. Primary open-angle glaucoma (POAG) constitutes the majority of all forms of glaucoma where the iris position is not affected. Traditionally, glaucoma has been viewed as a disease of elevated intraocular pressure (IOP). Excessive elevation of IOP can cause compression of retinal ganglion cell axons at the optic nerve head to affect axonal transport and alter the appropriate nutritional requirements for ganglion cell survival. Blood flow in the optic nerve head is also reduced because of compression of blood vessels and/or altered perfusion occurring when IOP is moderately elevated. Compelling evidence therefore exists to show that raised IOP can be the cause of visual loss in glaucoma. This is supported by the finding that the lowering IOP is often linked with the prevention of visual loss (Weinreb and Khaw, 2004).

A substantial number of glaucoma patients do not have raised IOP, however, and often lowering of elevated IOP does not result in the prevention of visual loss (Flammer and Orgül, 1998; Weinreb and Khaw, 2004). Moreover, not all ocular hypertensive patients have glaucoma. Clearly, raised IOP is not synonymous with loss of vision in all glaucoma patients. Factors other than raised IOP therefore contribute to loss of vision in glaucoma. However, unequivocal proof of risk factors other than raised IOP causing loss of vision in glaucoma remains a problem. A number of potential risk factors (Fig. 1) have been identified that include fluctuation of IOP, aging, family history, severe myopia, central cornea thickness, hypertension, hypotension, vasospasm, hemorheology, immune system, diabetes mellitus, sleep disturbances, family history, and light (Flammer et al., 2002; Pache and Flammer, 2006, Osborne et al., 2001, 2006). In addition, a number of candidate genes have also been associated with the risk of developing glaucoma (Abu-Amero et al., 2006).

Retinal ganglion cells and mitochondria

Retinal ganglion cell axons are unmyelinated within the ocular globe and have now been shown to have varicosities that are rich in mitochondria. These axons make desmosome- and hemidesmosome-like junctions with other axons and glial cells (Wang et al., 2003; Carelli et al., 2004). The mitochondria-enriched axon varicosities have been interpreted as functional sites with local

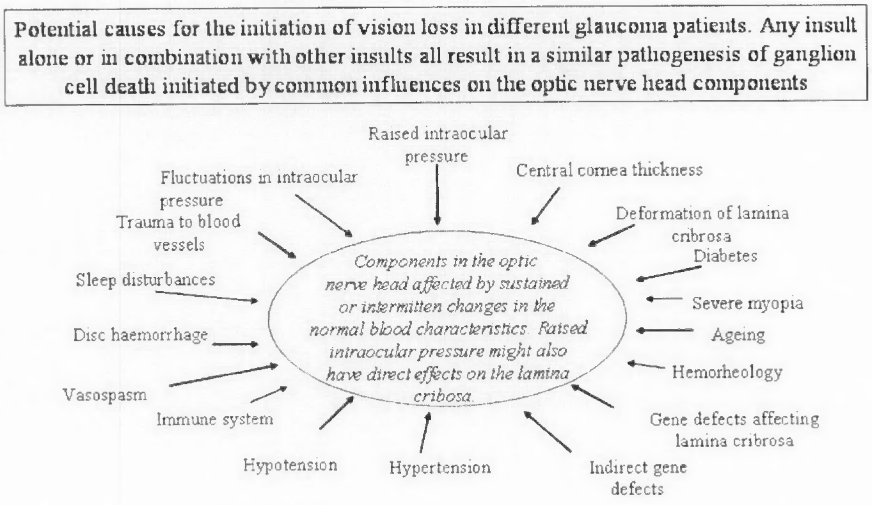

Potential causes for the initiation of vision loss in different glaucoma patients. Any insult alone or in combination with other insults all result in a similar pathogenesis of ganglion cell death initiated by common influences on the optic nerve head components
Raised intraocular pressure
Central cornea thickness
Fluctuations in intraocular pressure
Trauma to blood vessels
Deformation of lamina cribrosa
Diabetes
Components in the optic nerve head affected by sustained or intermitten changes in the normal blood characteristics. Raised intraocular pressure might also have direct effects on the lamina cribosa.
Sleep disturbances
Severe myopia
Disc haemorrhage
Ageing
Hemorheology
Vasospasm
Immune system
Gene defects affecting lamina cribrosa
Hypotension
Hypertension
Indirect gene defects

Fig. 1. Potential causes for an ischemic insult to the optic nerve head as might occur in glaucoma.

high-energy demand, possibly relevant for signal transmission. Significantly, immunocytochemical labeling of cytochrome c oxidase, a marker related to the intensity of oxidative phosphorylation, is greatest in the nerve fiber layer and prelaminar–laminar ganglion cell axons within the globe. It also decreases drastically in the myelin posterior to the lamina. Thus, there is an asymmetrical distribution of mitochondria along the ganglion cell axons, with the unmyelinated regions within the globe containing an enriched population of mitochondria (Bristow et al., 2002; Carelli et al., 2004). Clearly, an alteration in the functional status of these ganglion cell axon mitochondria will influence ganglion cell survival, and the question arises as to whether this phenomenon plays a part in the pathogenesis of optic neuropathies such as glaucoma.

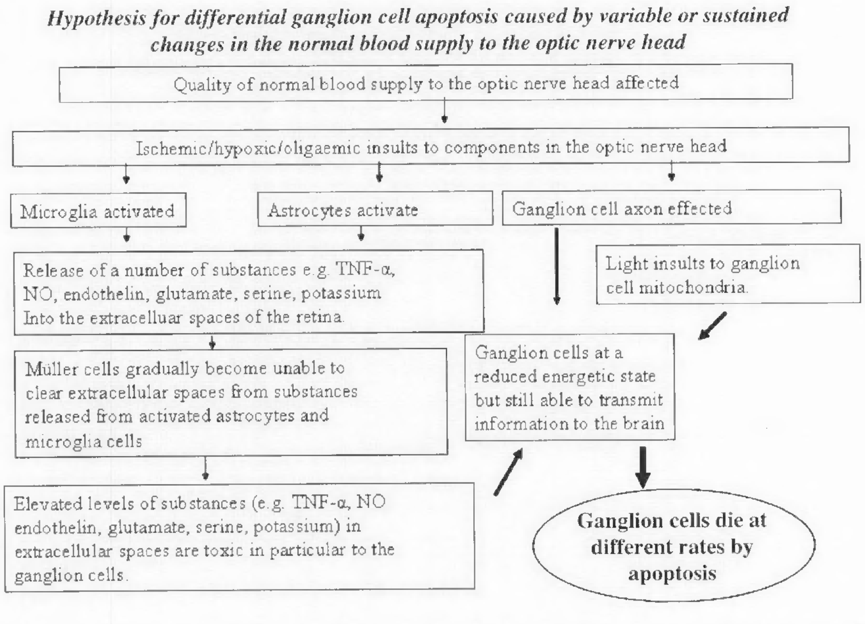
Mitochondria are constantly produced in neuronal soma and transported to their terminals where they participate in synaptic transmission and undergo fusion and fission (Chan, 2006). One explanation for the different number of mitochondria in the prelaminar and postlaminar regions of a ganglion cell axon is that mitochondria are transported more slowly in the prelaminar region as they are required to provide a lot of energy to propagate an action potential along an unmyelinated axon. If this is the case, then prelaminar axonal transport would vary between different ganglion cells depending on the length of unmyelinated axon within the globe. A reduction in nutrient/oxygen supply to the optic nerve head, for example, caused by raised IOP, will ultimately affect the efficiency of energy production by mitochondria in the prelaminar region, and as a consequence axonal transport is affected. There is good evidence to suggest that ganglion cell axonal transport is attenuated in glaucoma (Levy, 1976). Moreover, reduced nutrient/oxygen supply to the optic nerve head region might not affect all ganglion cells in the same way, with some being energetically compromised to a greater extent than others. This might be partially dependent on the length and number of mitochondria in a ganglion cell axon within the globe.

Recent studies have proven that mitochondrial respiratory chain enzymes such as favin and cytochrome oxidases are able to absorb light maximally around 440–450 nm, and as a consequence, contribute to the generation of reactive oxygen species (ROS) in cells (Chen et al., 1992, 2003; King et al., 2004; Godley et al., 2005; Osborne et al., 2006). The interaction of light with ganglion cell axon mitochondria in healthy cells probably results in a generation of ROS, which is removed by the appropriate scavenging mechanisms. However, when ganglion cells are in an energetic compromised state, normal scavenging mechanisms might not be adequate. The proposition has therefore been made that light becomes a risk factor to energetic compromised cells in glaucoma. Laboratory studies on cells in culture provide support for this notion (Osborne et al., 2006, 2008; Lascaratos et al., 2007).

Possible causes for ganglion cell death in glaucoma

It is now clear that the ganglion cell death in glaucoma is not solely caused by raised IOP. It is also evident that, in order to develop additional methods of treatment other than the lowering of IOP, it is necessary to understand the pathogenesis of the disease. From our present knowledge, it is tempting to conclude that impairment in the quality of blood flow in the microcirculation associated with the optic nerve head region is the factor initiating retinal changes that leads to loss of vision in glaucoma (Fig. 1), although it remains to be unequivocally demonstrated that optic nerve head blood flow is compromised in glaucoma. Present logic suggests that sustained or intermittent changes in the microcirculation in the optic nerve head region is likely to occur because of raised or fluctuation changes in IOP, aging, vasospasm, hemorheology, hypotension, or hypertension. This could result in variable ischemia/reperfusion and/or hypoxic insults to ganglion cell axons and glial cells in the optic nerve head region. This idea is supported by the finding that HIF-1α, activated by a reduced oxygen supply, is upregulated in pathological retinas from glaucoma subjects (Tezel and Wax, 2004).

How might ischemic insults to retinal ganglion cell axons and glia in the optic nerve head region cause the death of ganglion cells at different times in the life of a glaucoma patient? We have hypothesized that in the initial stages of the disease, ganglion cells will be forced to survive at a lower energetic status because of the altered optic nerve head blood flow (Fig. 2) and that as the disease progresses, individual ganglion cells will be at risk of a combination of different types of additional secondary insults. As a result, ganglion cells will die at different times. We have also proposed that secondary insults originate from light entering the eye (Osborne et al., 2006) and from altered function to glial cells (astrocytes, Müller cells, and microglia), originating from abnormal blood flow in the optic nerve head region that is also the cause for ganglion cells being energetically compromised (Figs. 1 and 2). This is based partly on experimental studies that have shown that a variety of potentially toxic substances (glutamate, TNF-α, serine, nitric oxide, and potassium) become elevated in the extracellular retinal spaces when retinal glial cell function is affected (see Tezel, 2006). Moreover, in pathological tissues, an upregulation of substances like nitric oxide synthase (involved in the formation of nitric oxide) (Neufeld et al., 1999) and TNF-α (see Tezel, 2006) has been reported. Also, an elevation of glutamate occurs in the vitreous humor of glaucoma patients (Dryer et al., 1996). Elevation of these substances will affect the survival of all retinal neurones by having membrane effects that may be receptor mediated. However, their effects on retinal ganglion cells will be particularly detrimental because these cells are in an already compromised energetic state. For example, elevated extracellular levels of glutamate will depolarize excessively all cells containing NMDA-type receptors, but the ganglion cells, being at a compromised energetic state, will not be able to compensate as efficiently as other neurones for this excessive depolarization, causing them to become dysfunctional and eventually die by apoptosis. This hypothesis therefore predicts that the trigger(s) for different ganglion cells dying by



Hypothesis for differential ganglion cell apoptosis caused by variable or sustained changes in the normal blood supply to the optic nerve head

Quality of normal blood supply to the optic nerve head affected
Ischemic/hypoxic/oligaemic insults to components in the optic nerve head
Microglia activated
Astrocytes activate
Ganglion cell axon effected
Release of a number of substances e.g. TNF-α, NO, endothelin, glutamate, serine, potassium Into the extracelluar spaces of the retina
Light insults to ganglion cell mitochondria
Müller cells gradually become unable to clear extracellular spaces from substances released from activated astrocytes and microglia cells
Ganglion cells at a reduced energetic state but still able to transmit information to the brain
Elevated levels of substances (e.g. TNF-α, NO endothelin, glutamate, serine, potassium) in extracellular spaces are toxic in particular to the ganglion cells.
Ganglion cells die at different rates by apoptosis

Fig. 2. Hypothetical cascade of events that lead to ganglion cells dying at different times during the lifetime of a glaucoma patient. The initial insult in the optic nerve head region affects all components (astrocytes, microglia, Müller cell end feet, ganglion cell axons, and lamina cribosa), resulting in ganglion cells existing at a reduced energetic state and an elevation of various substances (glutamate, TNF-α, NO, endothelin, d-serine, and potassium) in the extracellular space caused by activated astrocyte and microglia cells. These substances together with light impinging on mitochondria in ganglion cell axons eventually cause apoptotic insults to the energetically compromised ganglion cells at different times, depending on the axonal length and receptor profile of individual ganglion cells.

apoptosis in glaucoma will not necessarily be the same. This implies that the death pathways for different ganglion cells dying at various stages in glaucoma may not be the same.

Mitochondrial functions and apoptosis

Mitochondria are involved in numerous metabolic functions that include oxidative energy metabolism, the related production of ROS, and a role in promoting and regulating apoptotic death of the cell (Schon and Manfredi, 2003; Chan, 2006). Among the numerous stresses known to participate in mitochondrion-mediated apoptosis, at least in vitro, bioenergetic failure and elevated ROS figure prominently (Kroemer and Reed, 2000; Schon and Manfredi, 2003). Moreover, it is now established that light can be absorbed by mitochondrial photosensitizers, such as cytochrome oxidase and flavin-containing oxidases, causing a production of ROS (King et al., 2004; Godley et al., 2005). It is also known that the rate of ROS production from mitochondria is increased in a variety of pathologic conditions that include aging, hypoxia, and ischemia (Osborne et al., 1999; Chan, 2006; Morin and Simon, 2006).

Of key importance is the role of mitochondria in oxidative energy metabolism. Oxidative phosphorylation generates most of the cell's ATP, and any impairment of the organelle's ability to produce energy can have catastrophic consequences, not only due to the primary loss of ATP, but also due to indirect impairment of downstream functions, such as maintenance of organellar and cellular calcium homeostasis. Moreover, deficient mitochondrial metabolism will generate ROS that can wreak havoc in the cell. It is for such reasons that all evidence points to inadequate mitochondrial function being linked to apoptosis. This is supported by laboratory findings showing that substances that can maintain mitochondrial function (e.g., to allow ATP to be generated efficiently) or scavenge excessive ROS production blunt the process of apoptosis.

The hallmarks of apoptosis are condensation of nuclear and cytoplasmic contents, nuclear DNA fragmentation, cell blebbing, and autophagy of membrane-bound bodies. Apoptosis appears to occur via different pathways. A mitochondrion-mediated (intrinsic) pathway has been described where an external insult — elevated cytosolic calcium, to cite one example — acts to cause a release of cytochrome c, located in the inner membrane space of mitochondria. Cytosolic cytochrome c can then bind apoptotic protease-activating factor 1 (APAF-1), which then binds to the inactive form of caspase-9 (see Fig. 3). This complex "apoptosome" can then activate a cascade of events, which includes caspase-3, ultimately resulting in the hallmarks of apoptosis. Mitochondrion-mediated activation of caspase-9 can also occur via extracellular receptor-mediated signals (extrinsic pathway) to target various ligands (growth factor deprivation) — e.g., Bad, Bax, and Bik — to the mitochondrion, thereby causing cytochrome c release. Under certain circumstances, a separate mitochondrial-independent pathway also operates, which involves the activation of caspase-8 and caspase-3. In addition, a caspase-independent/mitochondrial-dependent pathway exists where apoptosis-inducing factor (AIF), confined to mitochondria, is released and translocates to the nucleus to cause chromatin condensation and DNA degradation (see Fig. 3).

Mitochondrial function enhancement and the attenuation of ganglion cell death

Mitochondria provide the bulk of a neurone's energy by oxidation of reducing equivalents (e.g., NADH and $FADH_2$), via the electron transport chain, to ultimately yield ATP. In addition, mitochondria contribute to cytosolic calcium buffering (Steeghs et al., 1997), apoptosis (Green and Reed, 1998; Kroemer and Reed, 2000; Schon and Manfredi, 2003), excitotoxicity (Peng et al., 1998), and generation of superoxide (McLennan and Degli Esposti, 2000). Also, mitochondrial flavin and cytochrome oxidases are affected by light to stimulate a production of ROS. Thus, an alteration in any aforementioned process or a combination of the processes maybe a cause of retinal ganglion cells dying in glaucoma.

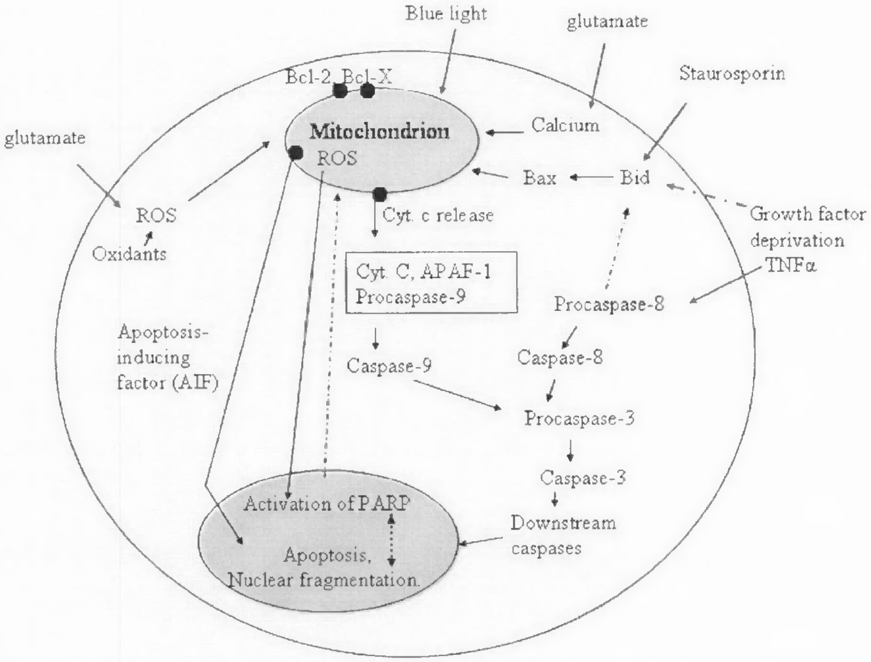


Fig. 3. Schematic view of the main mitochondrial-dependent and -independent (involves activation of caspase-8) apoptotic pathways. Mitochondrial-dependent apoptosis often involves the release of cytochrome c and the activation of caspase-9 but can also occur through mitochondrial release of cleaved AIF or ROS. AIF, apoptosis-inducing factor; PARP, poly(ADP-ribose) polymerase 1; ROS, reactive oxygen species; APAF-1, apoptotic protease-activating factor 1; Cyt. c, cytochrome c.

Substances that enhance mitochondrial function might therefore benefit glaucoma patients because ganglion cell axons have many mitochondria and these organelles are implicated in their apoptosis. Targeting impaired energy production is a realistic possibility. This will necessitate the use of an agent that can reach the retina, enhance the metabolic state of energetically compromised ganglion cells, and not affect healthy retinal cells adversely. Substances that might fulfil these criteria include creatine, α-lipoic acid, nicotinamide, and epigallocatechin gallate (EGCG), and each will be considered in turn. All four substances can be taken orally at regular intervals by humans without any obvious detrimental affects.

Creatine

Creatine is a guanidine compound that is ubiquitous among mammalian cells. Its concentration appears to reflect the energy requirements of various tissues, being highest in retinal photoreceptors with intermediate levels in the brain (Wyss and Kaddurah-Daouk, 2000). Creatine is released from the liver or consumed in the diet and transported into cells by specific transporters (Wyss and Kaddurah-Daouk, 2000). Within the cell, ATP and ADP and creatine are metabolized to form phosphocreatine by mitochondrial creatine kinase. Phosphocreatine then serves not only as an intermediate temporary energy buffer, but also as an energy shuttle from subcellular sites of energy production (mitochondria) to sites of energy consumption (Brewer and Wallimann, 2000). Thus, the phosphocreatine/creatine kinase system has been suggested to be of physiological importance in tissues with high energy and fluctuating energy requirements. This should apply to the intraretinal ganglion cell axons in particular because of their many mitochondria and presumed high level of creatine kinase. It has also been hypothesized that supplementation with creatine will result in cells making more phosphocreatine (Brewer and

Wallimann, 2000), and this has been supported by experimental studies. For example, creatine supplementation attenuates accumulation of oxidative stress markers and is shown to scavenge superoxide and peroxynitrite (Lawler et al., 2002). In cell culture experiments, creatine protects against toxicity induced by glutamate or β-amyloid (Brewer and Wallimann, 2000). Creatine supplementation has also been shown to exhibit remarkable neuroprotection in animal models of amyotropic lateral sclerosis (Klivenyi et al., 1998), Huntington's disease, Parkinson's disease, and traumatic brain injury (Zhu et al., 2004). Thus, the idea that creatine supplementation is beneficial for the treatment of glaucoma by attenuating the death process to ganglion cells seems to be a real possibility.

In preliminary studies, rats were injected subcutaneously twice a day with creatine (1 g/kg body weight/day) for six days, which was followed a day later for another five days (because of Sunday break from the laboratory). Controls received saline. On the sixth day, NMDA (concentration within the vitreous estimated to be 100 μM) was injected into the vitreous humor of one eye while the other received vehicle. On the 12th day, the animals were killed. Retinas were fixed and optic nerves analyzed by Western blotting for neurofilament light (NF-L) protein content relative to actin. Sections of fixed retinas were cut in areas of similar eccentricities. Eyes injected with NMDA showed a clear reduction of amacrine (ChAT) and ganglion (Thy-1) cell markers when compared with vehicle-injected eyes. However, in those animals that had been prophylactically treated with creatine, the effect of the NMDA injection was less evident (Fig. 4). Moreover, analysis of NF-L content in optic nerves showed that the reduction caused by NMDA treatment was significantly less in rats treated with creatine. These preliminary studies therefore provide support for the idea that creatine supplementation can attenuate an NMDA insult to inner retinal neurones, which include ganglion cells.

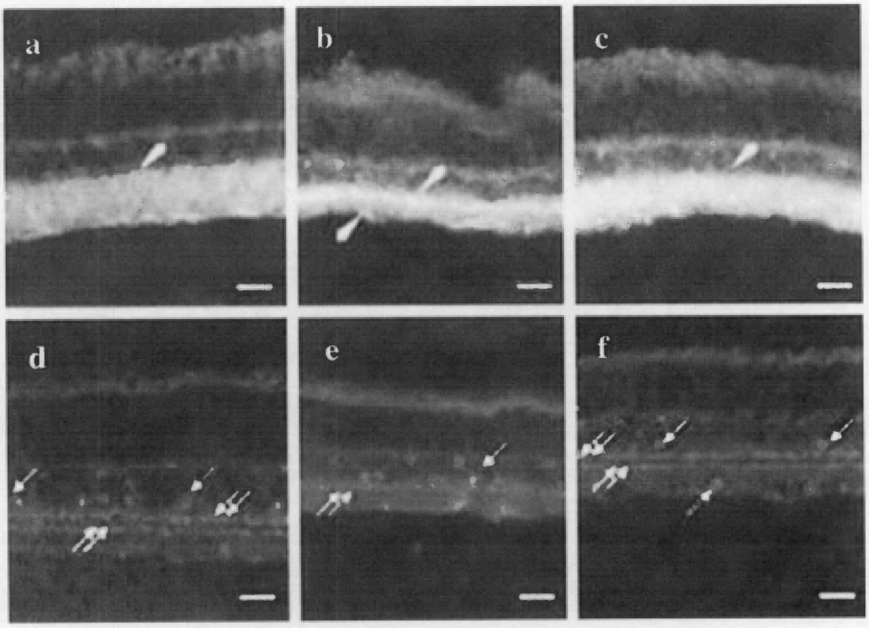


Fig. 4. Influence of creatine administration on the negative effects to the rat retina following an intraocular injection of NMDA. The normal thick band of Thy-1 immunoreactivity associated with the ganglion cells (a, arrow heads) and the typical localization of choline acetyltransferase (ChAT) associated with amacrine cells (small arrows) and their two processes in the inner plexiform layer (d) are much affected by NMDA injection (b, e). However, in animals treated with creatine (c, f), the influence of NMDA injection on Thy-1 and ChAT immunoreactivity is much reduced.

α-Lipoic acid

α-Lipoic acid (1,2-dithiolane-3-pentanoic acid) is a mitochondrial dithiol compound that functions as a coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. It is a cofactor in the mitochondrial dehydrogenase complex that catalyzes the oxidative decarboxylation of α-keto acids such as pyruvate and α-ketoglutarate. In the process, α-lipoic acid is reduced to dihydrolipoic acid and the two substances operate as a redox couple. Besides quenching of free radicals, lipoic acid/dihydrolipoic acid chelate transition metals and also assist in the regeneration of other antioxidants, such as glutathione, α-tocopherol, and ascorbate (Biewenga et al., 1997). Administration of α-lipoic acid to rodents has been demonstrated to reduce the damage that occurs after ischemic-reperfusion injuries in the cerebral cortex (Packer et al., 1997), heart (Freisleben, 2000), and peripheral nerve (Mitsui et al., 1999). α-Lipoic acid has also been found to exert protective effects in cell culture models of hypoxia and excitotoxicity (Tirosh et al., 1999). Despite such positive results, the effectiveness of α-lipoic acid as a neuroprotectant in the retina has largely been ignored to date. It is known that in the early diabetic rat retina, decreased free cytosolic and mitochondrial $NAD^+/NADH$ ratios and increased levels of 4-hydroxyalkenals, which are indicative of retinal hypoxia and increased lipid peroxidation, respectively, are reversed by treatment with α-lipoic acid (Obrosova et al., 2001). It is also important to note that α-lipoic acid is well tolerated by humans with no known side effects reported when taken daily (500–600 mg).

In order to test whether α-lipoic acid might be a candidate drug for the prophylactic use in attenuating ganglion cell death in glaucoma, we examined this possibility in a rat model given ischemia (Chidlow et al., 2002). Rats were injected intraperitoneally with either vehicle or α-lipoic acid (100 mg/kg) once daily for 11 days. On the third day, ischemia was delivered to the rat retina by raising the intraocular pressure above systolic blood pressure for 45 min. The electroretinogram (ERG) was measured prior to ischemia and five days after reperfusion. Rats were killed five or eight days after reperfusion, and the retinas were processed for immunohistochemistry and for determination of mRNA levels by RT-PCR. Ischemia –reperfusion caused a significant reduction in the a- and b-wave amplitudes of the ERG (Fig. 5), a decrease in nitric oxide synthase and Thy-1 immunoreactivities, a decrease in retinal ganglion cell-specific mRNAs (Thy-1 and NF-L), and an increase in bFGF and CNTF mRNA levels. All of these changes were clearly counteracted by α-lipoic acid. Moreover, in mixed rat retinal cultures, α-lipoic acid partially counteracted the loss of GABA-immunoreactive neurons induced by anoxia. The results of the study demonstrate that α-lipoic acid provides

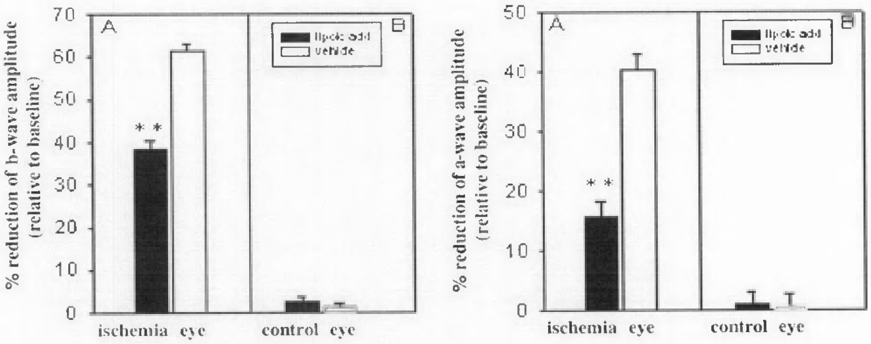


Fig. 5. Amplitude recordings of the a- and b-waves of electroretinograms from rats following ischemia/reperfusion and treated with either vehicle or α-lipoic acid. It can be seen that ischemia/reperfusion caused clear reductions in the a- and b-wave amplitudes, and these were significantly attenuated by α-lipoic acid. Results are means ± SEM, where $n = 16$.

protection from ischemia-reperfusion injuries to the retina as a whole and to ganglion cells in particular.

Nicotinamide

Nicotinamide is a precursor of NADH, which is a substrate for complex I (NADH-CoQ oxidoreductase). It is also an inhibitor of poly-ADP-ribose polymerase (PARP), an enzyme which is activated by DNA damage and in turn depletes both NADH and ATP (Eliasson et al., 1997). Activation of PARP plays a role in neuronal injury induced by both ischemia and MPTP toxicity (Mandir et al., 1999). Inhibition of PARP has also been shown to attenuate neuronal injury and ATP depletion produced by MTPT (Cosi and Marien, 1998). Appropriate nicotinamide pretreatment, in a middle cerebral artery occlusion model of stroke, reduced infarct volume, augumented cerebral blood flow, and increased brain NAD$^+$ levels in ischemic penumbra (Sadanaga-Akiyoshi et al., 2003). Moreover, in human studies, nicotinamide administration improved phosphocreatine/ATP ratios in certain patients. Recent studies on retinal cell cultures and rats support the supposition that appropriate nicotinamide supplementation might benefit glaucoma patients. In one series of experiments, nicotinamide (500 mg/kg) was administered intraperitoneally just before ischemia and into the vitreous (5 μl of a 200 μM solution) immediately

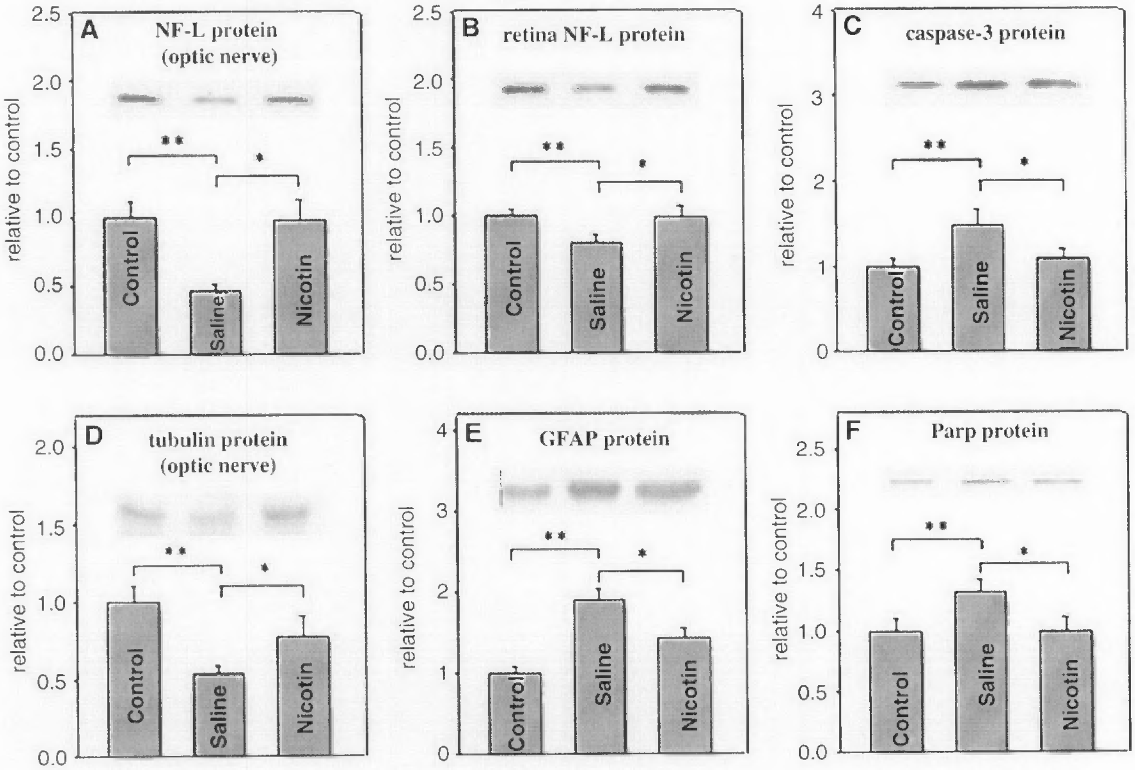


Fig. 6. Protein levels in whole retinas (NF-L, caspase-3, GFAP, and PARP) and optic nerves (NF-L and tubulin) relative to their individual amount of actin of eyes given ischemia where saline ($n = 6$) or nicotinamide ($n = 6$) was administered, and compared with the retina and optic nerve of the eye that in each case had not received any treatment (control). Results expressed as mean values ± SEMs. It can be seen that significant ($^{**}p < 0.01$) changes in all proteins were found in retinas given ischemia (saline) relative to the untreated control retinas. These changes in the retinas given ischemia are significantly blunted ($^{*}p < 0.05$) when the animals are treated with nicotinamide.

after the insult (Ji et al., 2008). Seven days after ischemia (IOP raised to120 mmHg for 50 min), retinas were analyzed for the localization of various antigens as well as the content of certain proteins and mRNAs. In addition, optic nerves were analyzed for their NF-L content. Ischemia/reperfusion affected a number of parameters in the retina that included a decrease in ganglion cell proteins (Thy-1 and NF-L) and mRNAs (NF-L and melanopsin) (see Fig. 6). Also, ischemia/reperfusion caused clear changes in the localization of nitric oxide synthase and ChAT and an activation of a certain protein/mRNAs (caspase-3, PARP) involved in apoptosis. In addition, ischemia/reperfusion caused a drastic loss of NF-L protein in the optic nerve. All these effects induced by ischemia/reperfusion were significantly blunted by nicotinamide treatment (Fig. 7).

Epigallocatechin gallate

A number of in vivo and in vitro reports have shown that catechins, a class of flavonoid present in large amounts in green tea, can exert neuroprotective actions in several models of neurodegenerative disorders (Mitscher et al., 1997; Mandel et al., 2005). The major catechin component of green tea, (–)-epigallocatechin-3-gallate (EGCG), at low micromolar and submicromolar concentrations, attenuates a variety of insults by what appear to be different mechanisms. For example, EGCG acts as an antioxidant, increases endogenous antioxidant defenses, counteracts inflammation-mediated neuronal injury, and causes a downregulation of pro-apoptotic genes. In relation to the eye, the antioxidant property of EGCG has been reported to account for its protective action of the lens against photooxidative stress

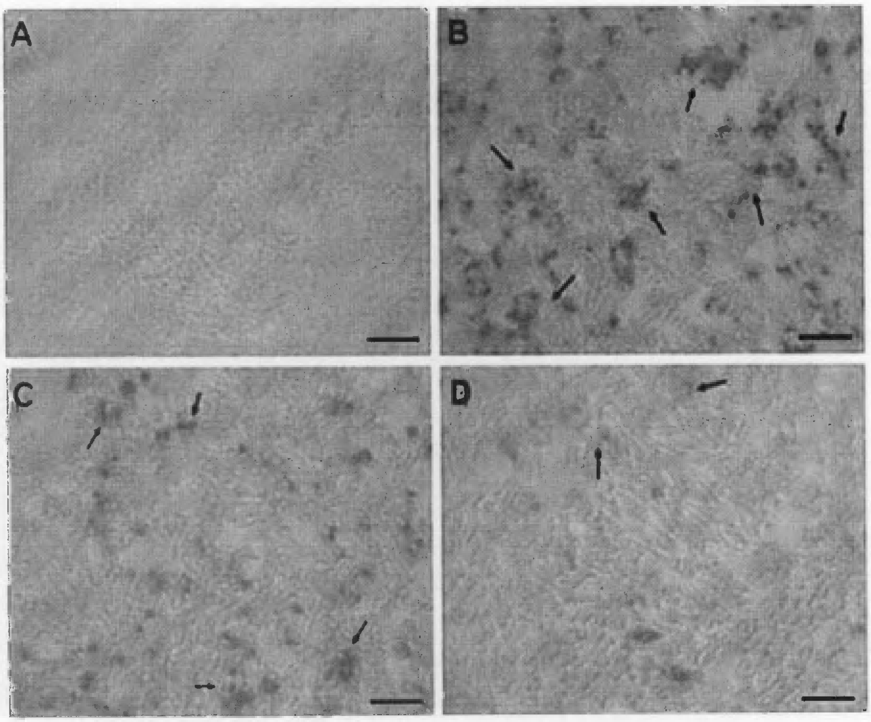


Fig. 7. Staining of RGC-5 cells exposed to light for 96 h (B) compared with cells maintained in the dark (A) by the APOP*ercentage*™ methodology for apoptosis (arrows). Inclusion of 0.5 mm nicotinamide (C) or 100 μM NU1025, a known PARP inhibitor (D), clearly reduced the positive staining of cell membranes as an indication for apoptosis induced by light. Scale bar = 20 μm. (See Color Plate 24.7 in color plate section.)

(Thiagarajan et al., 2001; Vinson and Zhang, 2005). Moreover, our recent studies have shown that photoreceptor degeneration induced by an intraocular injection of sodium nitroprusside (nitric oxide generator) can be attenuated by co-injection with EGCG (Zhang and Osborne, 2006) and that a systemic administration of EGCG attenuates retinal damage caused by ischemia/reperfusion (Zhang et al., 2007). These studies provide clear evidence that EGCG is a powerful antioxidant and can act as a neuroprotectant, making its potential use for the treatment of retinal degenerative diseases worthy of consideration.

Analysis of the bioavailability of EGCG in rats (Suganuma et al., 1998) shows orally administered EGCG to reach various tissues including the central nervous system, within 6 h. Moreover, a second administration after a 6 h interval enhances tissue levels four to six times above that of a single administration. In addition, all the evidence suggests that when a large amount of EGCG is orally administered, it is well tolerated with no ophthalmic abnormalities detected (Isbrucker et al., 2006). It has also been estimated that the half-life of green tea catechins after high oral dosing is between 451 and 479 min in albino rats, which is approximately 1–10 times longer than when administrated by an intravenous route (Zhu et al., 2000). In humans it has been shown that the pharmacokinetic parameters for EGCG are similar when present as decaffeinated green tea or as a pure form. Oral administration of EGCG at a high daily dose of 800 mg is known to be safe and without side effects in healthy individuals. Recently, we therefore examined whether orally administrated EGCG to rats can attenuate retinal ischemia (Zhang et al., 2008). Ischemia was delivered to one eye of a number of rats by raising the intraocular pressure, where EGCG (0.5%, each rat intake was approximately 200 ml/day) was present in the drinking water of half the animals three days before ischemia and also during the next five days of reperfusion. The ERGs of both eyes from all rats were recorded before ischemia and five days following ischemia. Seven days after ischemia, either retinas from both eyes of all rats were analyzed for the localization of various antigens or extracts were prepared for analysis for the level of specific proteins and mRNAs. Ischemia/reperfusion to the retina affected a number of parameters. These included the localization of Thy-1 and choline acetyltransferase, the a- and b-wave amplitudes of the ERG, the content of certain retinal and optic nerve proteins, and various mRNAs. Significantly, EGCG statistically blunted many of the effects induced by ischemia/reperfusion (see Figs. 8 and 9). This included ischemia-induced alterations to proteins associated with ganglion cells (NF-L and tubulin), Müller cells (GFAP), photoreceptors (rhodopsin kinase), and apoptosis (caspase-3, PARP, and AIF).

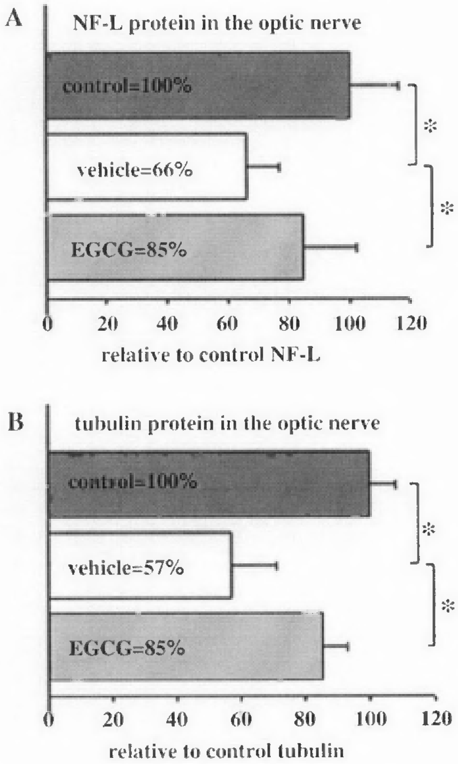


Fig. 8. NF-L (A) and tubulin (B) proteins relative to actin in the nonischemic optic nerves (control) and following ischemia/reperfusion with vehicle treatment (vehicle) or EGCG treatment (EGCG). Ischemia/reperfusion caused a significant decrease in the amounts of both NF-L and tubulin proteins. EGCG treatment significantly attenuated this reduction. Data are means with error bars indicating ±SEMs. $n = 8$ in each case, $^{*}p < 0.05$, and $^{**}p < 0.01$.

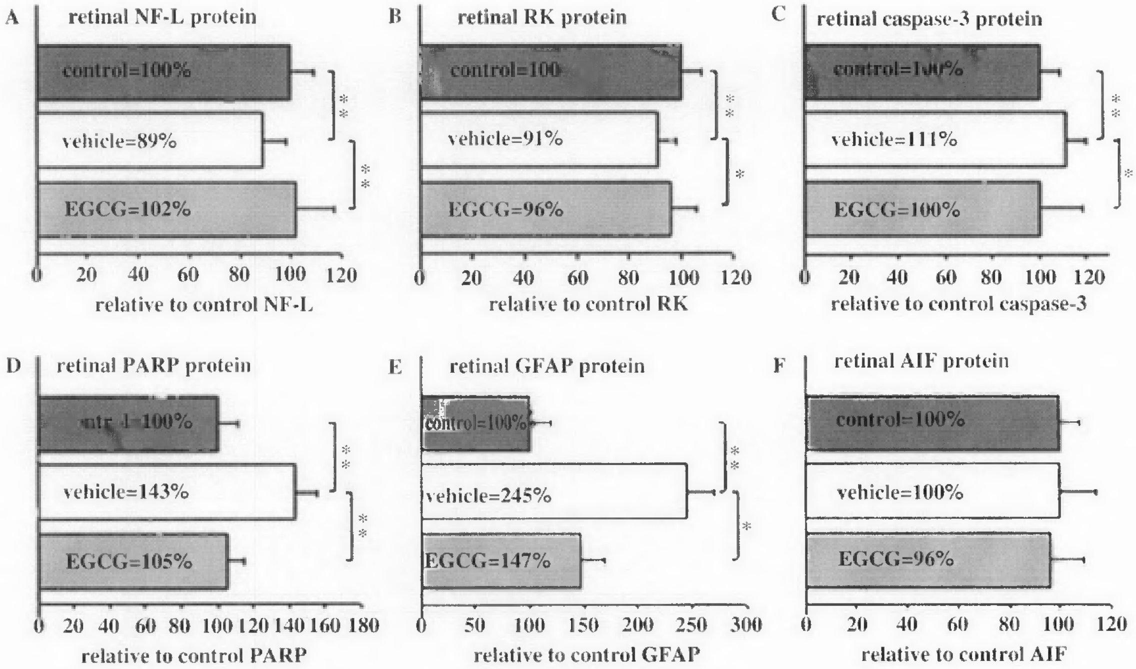


Fig. 9. NF-L (A), rhodopsin kinase (B), caspase-3 (C), PARP (D), GFAP (E), and AIF (F) proteins relative to actin in the retina in the nonischemic ischemia (control) and following ischemia/reperfusion with vehicle treatment (vehicle) or EGCG treatment (EGCG). Ischemia/reperfusion caused a significant change in the amounts of all proteins (except for AIF), and treatment with EGCG significantly counteracted this effect. Data are means with error bars indicating ±SEMs. $n = 6$ in each case, $^{*}p < 0.05$, and $^{**}p < 0.01$.

Conclusion

This article makes a case for the use of substances such as creatine, α-lipoic acid, nicotinamide, and EGCG as adjunctive therapies in the treatment of glaucoma. It is argued that, on diagnosis of glaucoma, immediate treatment with a substance that enhances mitochondrial function will increase the survival of still functional ganglion cells. It is postulated that the process of ganglion cell death in glaucoma occurs in a number of stages, initiated by an ischemic insult to their axons. In this state, ganglion cells can still survive for many years, but they are nevertheless more susceptible to further insults. The final demise of a particular ganglion cell occurs when the cell's energy metabolism reaches a critical point caused by additional insults. Potential additional insults include light or raised extracellular levels of glutamate, NO, or TNF-α. It is suggested that should a substance such as creatine, α-lipoic acid, nicotinamide, and EGCG be available to ganglion cells in their susceptible stage, mitochondrial function will be potentially enhanced and in the process prolong ganglion survival. The potential advantage of daily intake of such nontoxic compounds by glaucoma patients to slow down visual loss is a theoretical one based on animal studies but one that makes sense. It is not suggested as an alternative to the use of IOP-lowering agents but rather as a form of adjunct treatment.

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Effect of Systemic Creatine Monohydrate Supplementation on Denervated Muscle During Reinnervation: Experimental Study in the Rat

Ömer Özkan, M.D.,[1] Özgür Duman, M.D.,[2] Şenay Haspolat, M.D.,[2]
H. Ege Özgentaş, M.D.,[1] M. Bahadır Dikici, M.D.,[1] Inanç Gürer, M.D.,[3]
Hülya Aydın Güngör, M.D.,[4] and Ayşe Güzide Gökhan, M.D.[3]

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ABSTRACT

The purpose of this experimental study was to evaluate possible upgrading effects of systemic creatine monohydrate administration on the reinnervation of denervated muscle. At the same time, the protective effect of the agent on denervated muscle until ultimate reinnervation after nerve repair was quantified. The functional outcome of muscle reinnervation after creatine monohydrate application was compared with a control group. Forty adult Wistar rats weighing 180 to 220 g were used. The right sciatic nerve was dissected, exposed, and cut at the level of the midthigh in all rats. The experimental design consisted of two groups: experimental (animals were fed creatine monohydrate) and control (gavage feeding was provided by saline). Both groups were divided into two subgroups: subgroups A and B for the experimental group, and subgroups C and D for the control group. In subgroups A and C, the nerves were repaired with four 10-0 epineurial stitches. In subgroups B and D, both the proximal and distal ends of the nerves were ligated and no neural anastomosis was performed. In the experimental groups (subgroups A and B), the rats were fed by daily supplementation of oral creatine monohydrate, 300 mg/kg body weight. In the controls (subgroups C and D), oral supplementation was provided by saline.

Functional recovery was evaluated using walking track analysis, pinching test, and limb circumference and toe contracture measurements at the end of 6 months, after which the rats were sacrificed and nerve specimens from both ends of the repair sites and the whole gastrocnemius muscle were obtained to document the results of the histomorphometric and histochemical studies, including light microscopic examinations and muscle weight measurements. The mean functional recovery values in subgroups A, B, C, and D were 91 percent, 80 percent, 87 percent, and 59 percent, respectively. Functional recovery improved significantly in the experimental groups (in both the surgically repaired and unrepaired subgroups), compared with the control groups ($p < 0.05$). The pinching test revealed a statistically significant difference in nerve conduction between the experimental and control groups ($p < 0.05$). The limb circumference ratio of the surgically treated side to the untouched side in subgroups A, B, C, and D were noted as 0.95, 0.89, 0.91, and 0.87, respectively, and the difference between the experimental and the control groups was

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Journal of Reconstructive Microsurgery, Volume 21, Number 8, 2005; accepted for publication June 23, 2005. Address for correspondence and reprint requests: Ömer Özkan, M.D., Akdeniz Üniversitesi Hastanesi, Plastik ve Rekonstrüktif Cerrahi Anabilim Dali, B Blok kat 5, 07059 Antalya, Turkey. Department of [1]Plastic and Reconstructive Surgery, [2]Child Neurology, [3]Pathology, and [4]Neurology, Akdeniz University School of Medicine, Antalya, Turkey. Copyright © 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662. 0743-684X,p;2005,21,08,573,580,ftx,en;jrm01148x.

statistically significant($p < 0.05$). The differences between subgroups A and B, C and D, A and C, and B and D were also significant. The surgically repaired and creatine-supplemented subgroups demonstrated the best results in toe contracture index. The muscle weight measurement results were concordant with the results of the limb circumference ratio. In both surgically repaired subgroups (subgroups A and C), there were qualitatively significant amounts of myelinated fibers in the nerve distal to the anastomotic site; there were no myelinated fibers in the distal stumps of subgroups B and D. Histochemical analyses of the contents of the muscle fiber types also revealed no significant difference.

Overall, the results showed the useful effect of oral creatine supplementation on both surgically repaired and unrepaired nerve injuries. The best results were obtained from surgically repaired nerve injuries and also from the systemic creatine-supplemented subgroups. This study confirms that systemic administration of creatine monohydrate has a protective and upgrading effect on the functional properties of denervated muscle, especially in surgically reinnervated subjects.

KEYWORDS: Creatine monohydrate, denervated muscle, surgical reinnervation, rat model

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Although the management of nerve injury has dramatically changed in the past few decades, the current surgical and medical therapies for optimal desired functional restoration of severed peripheral nerves still result in imperfect clinical outcomes. Injuries to the nerves can be repaired with appropriate current surgical procedures, including primary nerve repair, autogenous nerve grafting, use of nerve conduit or allograft, cross-nerve grafting, microneurovascular tissue transfers, and split nerve transfer. Treatment can result in functionally predictable good outcomes, if basic surgical principles are employed. In addition, many medical agents have been proposed to enhance the efficacy of the surgical procedure. Among these are many growth hormones and factors that have been reported in the literature, both clinically and experimentally.[1–3]

Phosphocreatine is one of the muscle cell's major agents for maintaining its energy state.[4–6] Skeletal muscle takes creatine and phosphorylates it by creatine kinase. This serves as a source of ATP during some kinds of exercise. Dietary supplementation of creatine has been shown to increase the total creatine content of the muscle.[7–10] Physically, creatine monohydrate supplementation has resulted in modest improvements in muscular strength, endurance, and day-to-day activities for patients with neuromuscular disorders. It also increases lean body mass, high-intensity power output, and strength in young, healthy people.

The aim of this experimental study was to determine any possible upgrading effect of creatine monohydrate supplementation on denervated muscle that is surgically reinnervated. Its protective effects on muscle functional properties during the paralysis period until reinnervation was completed and on the functional and morphological recovery of the muscle were evaluated.

MATERIALS AND METHODS

A total of 40 adult Wistar rats weighing 180 to 220 g were used. At the beginning of the study, all the animals were selected from age-matched rats of the same species. The study was approved by the Animal Care and Use Committee of Akdeniz University. All animals were housed in standard environmental conditions, kept in rat cages, and offered standard rat chow and tap water. The animals were kept NPO the night before the procedure. No prophylactic antibiotic was administered. Intramuscular anesthesia of 90 mg/kg of ketamine hydrochloride and 10 mg/kg of xylazine were administered. A supplementary dosage of ketamine hydrochloride was given when necessary. The operative area was shaved with an electric shaver. The surgical procedure for nerve repair was performed using an operating microscope. The experiment consisted of two groups; experimental ($n = 20$) and control ($n = 20$). In all animals, after an oblique incision was made in the right gluteal region, the sciatic nerve was exposed through a gluteal muscle-splitting incision. It was dissected and mobilized from the sciatic notch to the branching point. The nerve was transected at the midlevel of these points with straight microscissors. In subgroups A and C ($n = 10$ each), the nerve was repaired with four 10-0 epineurial sutures. In subgroups B and D ($n = 10$ each), both the proximal and distal stumps of the nerve were ligated with 5-0 silk sutures and kept separate from each other to prevent any possible nerve sprouting and spontaneous neurotization,[11] and no anastomosis was performed.

In subgroups A and B, animals were fed by daily orogastric gavage feeding of 300 mg/kg creatine monohydrate with a 6 (Ch) no tube. In subgroups C and D, gavage feeding was provided by saline at 1 cc per day.

In all animals, a series of functional and histomorphometric tests were used to quantify nerve recovery and the functional outcome of muscle reinnervation.

Functional Evaluation *Walking Track Analysis* Walking track analysis, limb circumference measurement, and toe contracture index were used to evaluate functional recovery at 6 months post-surgery. Walking track analysis was performed using an apparatus with an 8.5- to 50-cm corridor opening onto a dark box in which a piece of paper had been placed on the bottom to obtain rat footprints, as described elsewhere.[11–13] The print lengths of both the surgically treated right side (EPL) and the untouched left side (NPL) were measured, and a print length factor (PLF) was then calculated as follows:

$$\mathrm{PLF} = (\mathrm{EPL}-\mathrm{NPL})/\mathrm{NPL}$$

From the print length factor, the percentage of functional recovery was calculated as:

$$\%\ \text{Functional Recovery}\ (\%\mathrm{FR}) = (1-\mathrm{PLF}) \times 100$$

Pinching Test The recovery of nerve function was analyzed by recording threshold stimulus. All recordings were carried out in a blind fashion. The animals were handled gently, and a small current generated by a stimulator (stimulus: 1 Hz, 0-1 mA) was applied to their hind feet. Thresholds, i.e., the lowest current to cause flexion of the feet, were recorded. A surface electrode was used for these recordings.

Limb Circumference Measurement The circumferences of the midthigh of both the operated and the untouched normal healthy sides were measured and compared. Results were given as the ratio of the surgically treated side to the untouched side.

Toe Contracture Measurement Toe contracture was measured at 6 months as described elsewhere.[13] Briefly, this was evaluated by grading the relationship of the toes to the axis of the foot at the metatarsophalangeal level. A straight appearance of this relationship was graded as normal (grade 1); when the motion of the toes was free and not locked and their tips were able to touch the ground, this was graded as mild contracture (grade 2); when the toes were again free but the tips did not touch the ground this was graded as moderate contracture (grade 3); and when the toes were locked to the sole, this was graded as severe contracture (grade 4).

Histomorphometric Evaluation At the end of the 6 months, the animals were sacrificed with an overdose of anesthetic, and gastrocnemius muscle and sciatic nerve specimens were taken to evaluate histomorphometric results. Histochemical analyses of the muscles were performed, as previously described elsewhere.[9,10] Briefly, muscle samples were lyophilized, cooled, and stored in liquid nitrogen until analysis. Samples were powdered and blood and fibrotic remnants were removed. A freezing microtome was used to obtain serial transverse sections (5 μm). These samples were stained for myofibrillar ATPase to identify the type of muscle fiber. Fiber types that exhibited high enzymatic reaction were classified as type I; those that exhibited intermediate reaction were classified as type IIA; and those that exhibited a poor reaction with large bands were classified as type IIB. The amount of each fiber was graded from 1 to 4 based on their intensity.

After routine tissue examination, peripherial nerve samples were cut in serial transverse section (5 μm), and dyed with myelin and trichrome. The presence of myelinated fibers, and the loss and degeneration of myelin in the peripherial nerve fibers were evaluated with myelin dye, and the presence of the stromal tissues between nerve fibers was examined with Masson's trichrome dye. Stromal tissue increase was classified as mild, moderate, or severe.

Muscle Weight Measurement The gastrocnemius muscles of both the operated and untouched sides were dissected from their origin to their insertion in all rats, and were weighed immediately with a sensitive scale. The muscle-weight value of the operated side was divided into the untouched side. Calculated rates were compared for each subgroup.

Statistical Analysis Statistical analysis of functional recovery was performed using the Kruskal-Wallis test. The Mann-Whitney U test was used to determine differences in muscle-weight measurement, and limb-circumference measurement between the experimental and control groups. The chi-square test was used for evaluation of toe contracture severity, and for evaluation of the results of the histochemical analyses of the muscles and nerves. A value of $p < 0.05$ was considered significant.

RESULTS

Functional Evaluation *Walking Track Analysis* The mean functional recovery values in subgroups A, B, C, and D were 91 percent, 80 percent, 87 percent, and 59 percent, respectively (Table 1). The difference between the experimental (subgroup A and B) and control (subgroup C and D) groups was statistically significant ($p < 0.05$). Although both the difference between subgroups A and B, ($p < 0.05$), and that between subgroups C and D were significant, ($p < 0.05$), no significant difference was obtained between subgroups A and C. The difference between subgroups B and D was statistically meaningful ($p < 0.05$).

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Table 1 Mean Values of Functional Recovery Rate, Limb Circumference Ratio, Toe Contracture, and Muscle Weight Measurement Ratio of Subgroups at the End of 6 Months

Subgroups	Functional Recovery (%)	*Pinching Test (mean current to cause flexion of the feet)(mA)*	Limb Circumference Ratio (experimental side/ untouched side)	Toe Contracture Severity (see text)	Muscle Weight Measurement Ratio (experimental side/untouched side)
A(n = 10)	91 ± 14	2.52 ± 0.27	0.95 ± 0.05	1.9 ± 0.74	0.87 ± 0.08
B(n = 10)	80 ± 16	5.28 ± 0.38	0.89 ± 0.06	2.9 ± 0.57	0.42 ± 0.18
C(n = 10)	87 ± 20	3.26 ± 0.70	0.91 ± 0.06	2.4 ± 0.52	0.69 ± 0.10
D(n = 10)	59 ± 33	6.28 ± 0.34	0.87 ± 0.05	3.3 ± 0.67	0.20 ± 0.02

mA: miliampere

Pinching Test The mean currents to cause flexion of the feet in subgroups A, B, C, and D were recorded as 2.52 mA, 5.28 mA, 3.26 mA, and 6.28 mA, respectively (see Table 1). The test revealed significant differences between subgroups A and C ($p < 0.05$), and between subgroups B and D ($p < 0.05$). The differences between subgroups A and B ($p < 0.05$), and those between subgroups C and D ($p < 0.05$) were also statistically significant.

Limb Circumference Measurement The ratios of the surgically treated side to the untouched side in subgroups A, B, C, and D were noted as 0.95, 0.89, 0.91, and 0.87, respectively (see Table 1). The difference between the experimental (A and B) and the control (C and D) groups was statistically significant ($p < 0.05$). Although no significant difference was observed between subgroups B and D, the difference between A and C was significant ($p < 0.05$). While the difference between subgroups A and B was significant ($p < 0.05$), no meaningful significance was obtained between subgroups C and D.

Toe Contracture Measurement The mean toe contracture indexes in subgroups A, B, C, and D were 1.9, 2.9, 2.4, and 3.3, respectively (see Table 1). The difference between subgroups A and C was statistically significant ($p < 0.05$). No significant difference was seen between subgroups B and D. The least severe toe contracture results were in subgroup A, comprising surgically repaired and systemic creatine-administered rats.

Histomorphometric Evaluation *Light Microscopic and Morphometric Examination* In the surgically repaired subgroups (subgroups A and C), there were qualitatively significant amounts of myelinated fibers in the nerve distal to the anastomotic site (Fig. 1). There were no myelinated fibers in the distal stumps of subgroups B and D.

The amount of stromal tissue between the nerve fibers was mild in subgroups A and C, but there was a significant increase in stromal tissue in the nerve fibers in subgroups B and D.

The mean intensity of type I fiber in subgroups A, B, C, and D was graded as 2.8, 2.6, 1.8, and 1.3, respectively; the intensity of type IIA fiber in each group was graded as 2.8, 2.5, 1.7, and 1.4; and the intensity of type IIB fiber was noted as 3.5, 3.3, 2.3, and 1.9, respectively (Table 2). The quantity of all fiber types

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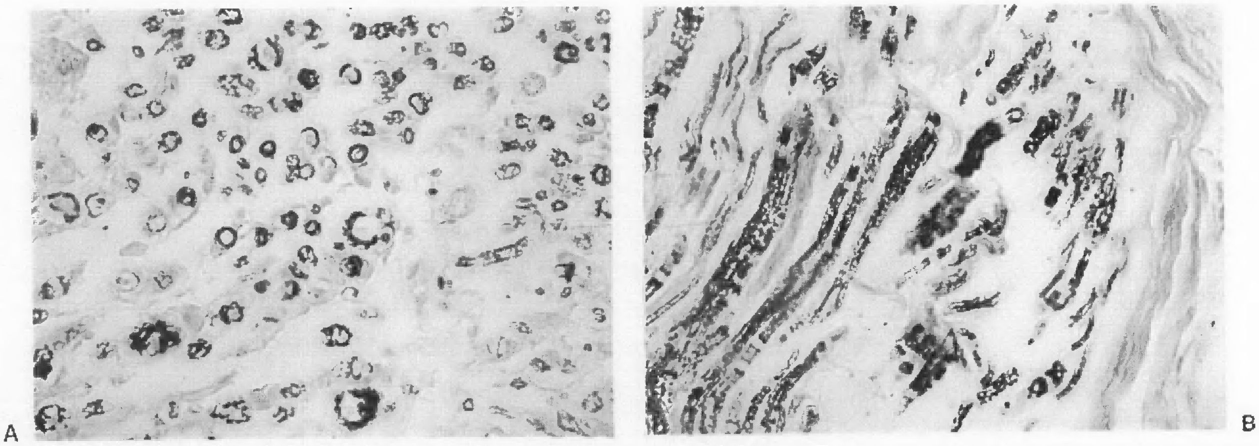

Figure 1 Morphometric evaluation of the nerve fibers in the surgically repaired and creatine monohydrate-supplemented subgroup showing significant amounts of myelinated fibers in the nerve distal to the anastomotic site. (A) Transverse section. (B) Longitudinal section. (myelin dye, original magnification ×200)

Table 2 Mean Intensity of Muscle Fibers in Subgroups (see text)

Subgroups	Type I	Type IIA	Type IIB
A(n = 10)	2.8	2.8	3.5
B(n = 10)	2.6	2.5	3.3
C(n = 10)	1.8	1.7	2.3
D(n = 10)	1.3	1.4	1.9

was significantly increased in the creatine monohydrate-supplemented subgroups, compared with the other subgroups ($p < 0.05$) (Fig. 2).

Muscle Weight Measurement The mean weight ratios of the operated to the untouched sides of the gastrocnemius muscle in subgroups A, B, C, and D were 0.87, 0.42, 0.69, 0.20, respectively (see Table 1). There were statistically significant differences in muscle weight between subgroups A and C ($p < 0.05$), B and D ($p < 0.05$), A and B ($p < 0.05$), and C and D ($p < 0.05$).

DISCUSSION

Peripheral nerves can be damaged in a number of ways, including by iatrogenic surgical factors, infiltration by tumors, accidents, inflammatory processes, or external physical agents such as traction, pressure, or stretching. If transected nerves remain unrepaired, substantial muscle atrophy and downregulation of motor endplates will result in inferior clinical outcomes. The patterns of muscle paralysis vary in terms of the degree and duration of nerve damage. Totally paralyzed muscle can never be returned to normal by any of the current nerve repair techniques, and it has been clearly established that autogenous regeneration of the peripheral nerve stump cannot occur. Although nerve coaptation techniques have improved with advances in microsurgical skills, primary nerve repair—either epineurial or fascicular—still remains the first choice of repair.[11] In addition to surgical interventions, many medical agents such as growth hormones and factors have been proposed to enhance repair efficacy in recent decades.[1–3] The importance of the application of nerve growth factors to establish successful nerve regeneration has been mentioned in many reports. However, effective and certain applications and mechanisms still remain unclear.

Injuries to the nerves should be repaired with the appropriate surgical procedure. Although no surgical technique has been described to accomplish the optimal desired results to date, a primary nerve repair should be performed when surgical and clinical conditions allow. In this way, providing a healing process between the two injured stumps by traveling axon sprouts from the proximal to the distal and finally to the end organs, the optimal condition for nerve healing may be possible. Although many different surgical techniques have been described as achieving better coaptation, none of them has been proven to possess advantages over the conventional epineurial primary nerve repair technique.

Skeletal muscle is a highly reactive tissue in both normal and pathological situations.[11,14] Muscle fibers adapt to changes by increasing or reducing the number of contractile units. If the nerve of the muscle that represents the source of the neural input is transected, the muscle fiber undergoes a series of atrophic changes. If the denervation period lasts too long, this will result in ultimate disappearance.

Muscle disuse due to nerve injury results in muscle atrophy and reduced muscle functional capacity, and may impair future day-to-day activities. After proper nerve repair, the time taken for reinnervation of the responsible muscle will depend on the nerve regeneration process. Various strategies have been proposed to prevent or reverse this disuse atrophy and final functional impairment during the regeneration period. Oral creatine supplementation has been suggested as an effective intervention to prevent or reverse neuromuscular degeneration in humans.[15–23]

Dietary supplementation with creatine has been shown to increase total creatine concentration in muscle,

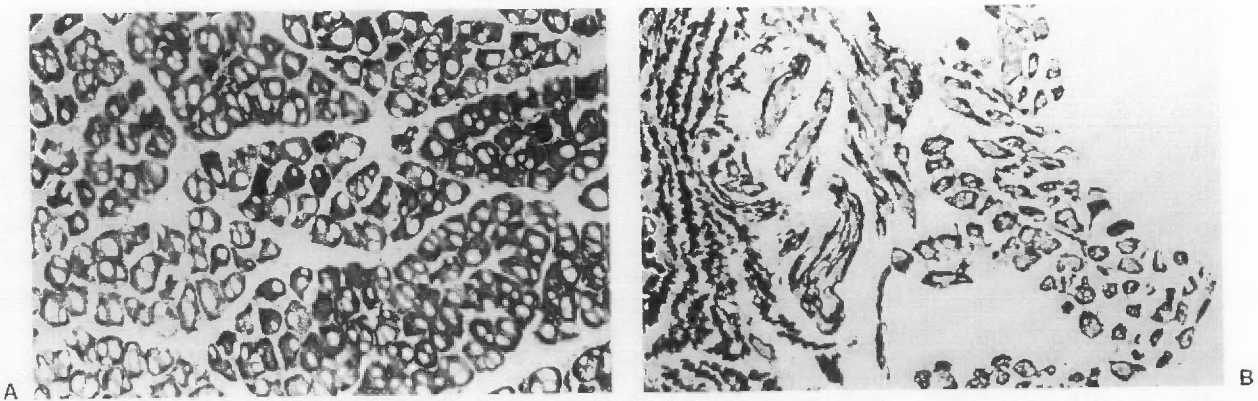

Figure 2 Histomorphometric evaluation of the muscles (A) Experimental group (B) Control group. The quantity of all types of fibers was found to be significantly increased in the creatine monohydrate-supplemented subgroups, compared with the others (myofibrillar ATPase, NADH stain, ×100).

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so that it acts on the energy state in the cell in both humans and rats.[4–10] It is a natural nutrient found in animal foods and is suggested to be an effective nutritional agent to enhance exercise performance. Oral intake of creatine has been found to improve muscle functional capacity in patients with various forms of neuromuscular pathologies. Creatine supplementation has not been prohibited for athletes, so its use is currently regarded as legal. Short-term and even long-term creatine supplementation of up to 8 weeks has not been associated with any major health risk.[18] Chronic creatine monohydrate supplementation increases lean body mass, high-intensity power output, and strength in young healthy people.[1–4] In experimental and clinical studies, it has been proven that oral creatine can stimulate muscle hypertrophy. It has also been demonstrated that oral creatine supplementation is an effective agent for both functional and structural adaptation of skeletal muscle during the disuse atrophy period. Creatine monohydrate supplementation has demonstrated modest improvements in muscular strength, endurance, and activity in patients with neuromuscular disorders.

The present study was carried out to evaluate the effect of oral creatine supplementation on muscle rehabilitation during the reinnervation period after surgical repair of the severed nerve in an animal model. The rat is a preferred animal in experimental studies because it is inexpensive, readily available, and reliable. End-to-end epineurial anastomosis was performed between the proximal and distal stumps of the severed sciatic nerve. During the reinnervation period, oral supplementation of creatine monohydrate was administered to protect the functional and structural capacity of the target muscles. Reinnervation of the paralyzed muscle and the protective effect of the creatine were measured, and compared with those of control groups and normal untouched muscles.

Hespel et al.[10] demonstrated that oral creatine supplementation of 12 weeks duration increased muscle functional capacity during the disuse atrophy period. They suggested that in patients with neuromuscular pathologies, creatine supplementation induced muscle hypertrophy and increased muscle functional capacity in the absence of a regular rehabilitation program. In the current study, functional recovery of the reinnervated muscle was found to be superior in the creatine monohydrate-supplementation group, compared with the control group. The structural and functional properties of the muscle were protected and improved with creatine intake during the reinnervation period. Functional evaluation studies showed that the most efficient results were obtained from subgroup A, comprising nerve repaired and oral creatine-supplemented rats.

The results of the qualitative evaluation of peripheral nerve fibers revealed no significant effect of creatine supplementation. Thus, the basic effect of creatine monohydrate can be attributed to the muscle tissue. Creatine supplementation increased muscle-fiber diameter to a similar degree in types I, IIA, and IIB fibers. As reported by Persky et al.[9] and Hespel and colleagues,[10] this indicates that histologically individual muscle-fiber distribution may not play a critical role in the effects of creatine monohydrate.

The protective effect of creatine monohydrate supplementation during the reinnervation period is also evident. Jacobs et al.[24] demonstrated that creatine monohydrate supplementation enhances upper extremity work capacity in patients with cervical cord injuries. In the present study, the muscle-weight measurements were in agreement with the functional studies. During the reinnervation period, muscle atrophy can be prevented by systemic creatine monohydrate supplementation. The hypertrophic effect of creatine has been observed in all systemically supported individuals.

In conclusion, the current study demonstrates the efficacy of oral creatine supplementation in stimulating muscle hypertrophy and enhancing the functional capacity of the denervated muscle during the reinnervation period. The results confirmed that oral creatine monohydrate supplementation is a simple and reliable method to protect and maintain the morphological structure of the muscle during this disuse period. It should be evaluated with similar clinical indications.

ACKNOWLEDGMENT

We thank the Akdeniz University Scientific Research Projects Unit for their support of this study. Materials in this paper were presented at the Seventh International Congress, Mediterranean Society of Myology, Kusadasi, Turkey, 2004.

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INTRINSIC DISSOLUTION PROFILES OF CREATINE NITRATE, CREATINE MONOHYDRATE AND BUFFERED CREATINE

Ambrish Pandit[1], Pinal Mistry[1], Pat Dib[2], Alexander Nikolaidis[3] Alekha K. Dash[1]
Department of Pharmacy Sciences, Creighton University, Omaha, NE 68178. Department of Physiological Science UCLA. Aristotle University Of Thessaloniki.

Objective: The objective of this study was to determine the dissolution characteristics of three different forms of commercially available creatine including creatine nitrate (CN), creatine monohydrate (CM) and buffered creatine (BC) under different temperature and pH. **Methods:** Intrinsic dissolution studies were carried out at 37°C and room temperature in pH 2.5 and 7.4 buffer using modified Wood's apparatus. CN, CM and BC samples (~0.5 g) were compressed in the dies with constant surface area of 1.21 cm^2 using a Carver press at 2000 psi with a dwell time of 10 sec. These dies were placed in the USP dissolution apparatus (type II) containing 140ml of dissolution media, with paddle speed of 50 rpm. Dissolution medium were collected at definite time intervals over a period of 3 hours for CN and 7 hours for both CM and BC and analyzed for creatine using a validated HPLC method. **Results:** A plot of the amount of creatine dissolved (mg)/surface area (cm^2) versus time gives the slope (k') which is directly proportional to the dissolution rate constant. The k' (mg/cm^2 hr) values for CN, CM and BC in pH 2.5 buffer at room temperature were 293.0±3.48, 59.1±2.06 and 71.1±0.59. At 37°C these k' (mg/cm^2 hr) values were 327.3±2.66, 97.9±0.88 and 117.9±1.12, respectively. In pH 7.4 buffer, the k' (mg/cm^2 hr) values for CN, CM and BC were 278.9±14.6, 52.1±0.22 and 50.6±0.95 at room temperature and 319.5±14.3, 86.0±5.05 and 96.9±9.28 at 37°C, respectively. **Conclusions:** The dissolution rates of all three creatine forms were higher at 37°C as compared to at room temperature. CM and BC showed a higher dissolution rate at pH 2.5 than that at pH 7.4, irrespective of temperature. However, the dissolution rate of CN was not affected by the pH of the dissolution media. Comparison of the dissolution profiles for these three creatine forms clearly indicated that CN has the higher intrinsic dissolution rate constant as compared to CM and BC irrespective of temperature and pH of the dissolution media. Results of this study further predict a better bioavailability of CN as compared to CM and BC. Partially funded by ThermoLife International.

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ISBN: 1-56363-364-7

Zinc: Excessive use of zinc may cause decreased absorption of copper. One explanation for this interaction is that high dietary zinc induces intestinal metallothionein. Copper has a stronger affinity for intestinal metallothionein than does zinc and displaces zinc in intestinal metallothionein and is trapped. Copper depletion was observed when supplements of 50 milligrams or more of zinc were given for extended periods. Copper absorption was not affected by zinc doses of 16.5 milligrams daily for extended periods.

Vitamin C: Vitamin C supplementation of 1,500 milligrams daily caused the activity of the copper transporting protein ceruloplasmin to decline. Vitamin C supplementation of 600 milligrams daily also caused a decline in ceruloplasmin, but copper absorption was not impaired.

FOODS

Concomitant intake of copper with foods rich in phytic acid (unleavened bread, raw beans, seeds, nuts and grains and soy isolates) may decrease the absorption of copper.

Diets high in fructose may decrease copper status.

OVERDOSAGE

Gram amounts of copper can cause coma, oliguria, hepatic necrosis, vascular collapse and death.

DOSAGE AND ADMINISTRATION

Copper supplements are available in several forms, including cupric oxide, copper gluconate, copper sulfate and copper amino acid chelates. Copper supplements are typically found in combination products. Typical doses range from 1.5 to 3.0 milligrams daily. There is no reason to use higher amounts of copper.

The Food and Nutrition Board of the U.S. National Academy of Sciences has recommended the following estimated safe and adequate daily dietary intake (ESSADI) values for copper:

Age (year)	ESSADI (milligrams)
Infants	0.4-0.7
1-3	0.7-1.0
4-6	1.0-1.5
7-10	1.0-2.0
11-18	1.5-2.5
Adults	1.5-3.0

HOW SUPPLIED

Capsules — 5 mg

Solution

Tablets — 2 mg, 5 mg

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Creatine

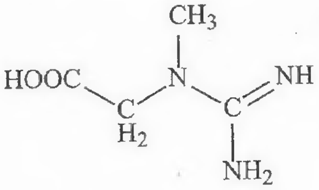
TRADE NAMES

Creatine is available generically from numerous manufacturers. Branded products include Muscle Power (Mason Vitamins), Creatine Fuel (Twinlab), Creatine Booster (Champion Nutrition), Creatigen (Bricker Labs), CreaVate (Prolab Nutrition), Perfect Creatine (Nature's Best), Xtra Advantage Creatine Serum (Muscle Marketing USA), Micronized Creatine (Met-Rx), Creavescent (GEN), Power Creatine (Champion Nutrition), Phosphagen (GNC), Crea-Tek (Iron Tek), Effervescent Creatine Elite (Muscle Link).

DESCRIPTION

Creatine is a non-protein amino acid found in animals and, in much lesser amounts, plants. Creatine is synthesized in the kidney, liver and pancreas from the amino acids L-arginine, glycine and L-methionine. Following its biosynthesis, creatine is transported to the skeletal muscle, heart, brain and other tissues. Most of the creatine is metabolized in these tissues to phosphocreatine (creatine phosphate). Phosphocreatine is a major energy storage form in the body.

Creatine is known chemically as N-(aminoiminomethyl)-N-methyl glycine and its structural formula is:



Creatine

Supplemental creatine is typically a synthetic substance. It is a solid and is water-soluble.

ACTIONS AND PHARMACOLOGY

ACTIONS

Supplemental creatine may have an energy-generating action during anaerobic exercise and may also have neuroprotective and cardioprotective actions.

MECHANISM OF ACTION

Since the action of supplemental creatine has yet to be clarified, the mechanism of action is a matter of speculation. Much is known, however, about the biochemistry of endogenous creatine. Creatine is mainly synthesized in the kidney, liver and pancreas. In its synthesis, the guanidino group of L-arginine is transferred to glycine to form guanidinoacetate and ornithine by a transamidinase reaction, a reaction that takes place in the pancreas, liver and kidney. Guanidinoacetate is methylated by S-adenosylmethione (SAMe) to form creatine. About 1 to 2 grams of creatine are biosynthesized daily and another 1 to 2 grams are obtained from diet.

In muscle and nerve, most of the creatine is phosphorylated to phosphocreatine (PCr) in a reaction that is catalyzed by the enzyme creatine kinase (CK). There are three isoforms (isoenzymes) of CK. CK-MM is the skeletal muscle isoform; CK-BB, the brain isoform, and CK-MB, the isoform found in cardiac muscle. Most of the PCr in the body is in skeletal muscle.

Creatine, creatine kinase and phosphocreatine make up an intricate cellular energy buffering and transport system connecting sites of energy production in the mitochondria with sites of energy consumption. CK is a key enzyme involved in cellular energy homeostasis. It reversibly catalyzes the transfer of the high-energy phosphate bond in PCr to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP), and it catalyzes the transfer of the high-energy phosphate bond in ATP to creatine to form PCr. During periods of intense exercise and skeletal muscle contraction, bioenergetic metabolism switches from one in which oxidative phosphorylation is the major pathway of ATP production to one in which so-called anaerobic glycolysis becomes dominant. Much less ATP would be generated during this period if it were not for phosphocreatine (PCr) being the only fuel available to regenerate ATP during this period. Thus the availability of PCr is the limiting factor of skeletal-muscle performance during high intensity and brief bursts (about 10 seconds) of activity. Supplemental creatine may increase PCr levels in skeletal muscle and hypothetically enhance ATP turnover during maximal exercise.

Creatine supplementation of transgenic amyotrophic lateral sclerosis (ALS) mice carrying the superoxide dismutase (SOD)1 mutation has reportedly produced improvement in motor performance and extension of survival, as well as protection against loss of both motor neurons and substantia nigra neurons. Mitochondrial dysfunction is among the earliest features found in these mice models of familial ALS. Creatine administration to these mice appears to stabilize mitochondrial CK and inhibits opening of the mitochondrial transition pores.

Creatine, as well as a creatine analogue called cyclocreatine, inhibit growth of a broad range of solid tumors in rat models of cancer; these tumors express high levels of CK. Although the mechanism of tumor inhibition is unknown, there is speculation about what it may be. Creatine feedback inhibits the transamidination step in its biosynthesis. This results in sparing L-arginine, the limiting precursor in creatine synthesis. More available L-arginine can lead to increased levels of nitric oxide (NO), which is a factor in macrophage activation. Another possibility is that glycolysis is inhibited in these tumors. Phosphocreatine inhibits enzymes in the glycolitic pathway, including glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase and pyruvate kinase.

PHARMACOKINETICS

Creatine is absorbed from the small intestine and enters the portal circulation and is transported to the liver. The ingested creatine, along with creatine made in the liver, is then transported into the systemic circulation and distributed to various tissues of the body, including muscle and nerves, by crossing the cell membrane via a specific creatine-transporter system against a 200:1 gradient. Chronic creatine supplementation in rats down-regulates creatine transporter protein expression. If this is also the case in humans, then chronic creatine supplementation would lead to lower amounts entering cells at any given time.

Within muscle and nerve cells, about 60 to 67% of the creatine entering the cells gets converted to phosphocreatine via the enzyme creatine kinase. About 2% of creatine is converted to creatinine, and both creatine and creatinine are excreted by the kidneys.

INDICATIONS AND USAGE

There is some evidence that supplemental creatine may enhance performance in a limited number of high-intensity, short-term physical activities, but the data are mixed, and no ergogenic effect has been convincingly demonstrated outside of laboratory settings. Adequate safety data are still lacking. There is some very preliminary data that creatine may be helpful in treating muscular dystrophy and amyotrophic lateral scleroses and may improve skeletal muscle function in some with congestive heart failure and gyrate atrophy of the retina. Creatine has inhibited the growth of some solid tumors in rats, but no human cancer data exist.

RESEARCH SUMMARY

Limited muscle function benefit has been noted in some early studies of creatine. All of these studies have been of short duration (mostly lasting one or two weeks and, in no case, more than eight weeks). Many other studies have found no benefit.

A recent review article summarized the results of 71 trials published between 1993 and 1997. Of those that studied effects of supplemental creatine (usually 20 grams daily for 4 to 21 days) on short term, high-intensity performance, 23 reported positive effects and 20 reported no effect. Studies examining the effects of creatine on oxidative energy systems, muscle isokinetic torque and isometric force produced similarly mixed results. Among the few field tests that have been conducted (all related to swim sprints) none detected any effect on athletic performance. Only among studies of cycle ergometer performance was there any superiority of creatine over placebo (11 trials reported improvement, while six others reported no improvement).

Since this review was published there have been a few more positive than negative reports, but, again, the positive effects are almost entirely seen in laboratory settings and are confined to short-term, high-intensity performance.

One author recently reviewed the creatine data and has concluded that supplemental creatine achieves an ergogenic effect, at least in the laboratory, in repeated stationary cycling sprints. But he found no convincing evidence that it does so in single sprints. He also discerned a possible ergogenic effect in weightlifting, but none in running or swimming sprints of any kind. He and others have speculated that the weight gain that typically accompanies creatine supplementation offsets any ergogenic effect that might otherwise benefit runners and swimmers.

Some have claimed that this weight gain, typically 0.5 to 1.6 kilograms occurring in the first few days to first two weeks of creatine supplementation, is evidence of increased muscle mass. Most researchers, however, believe that this weight gain is accounted for by creatine-induced water retention. The longer-term studies needed to confirm or refute claims that chronic creatine supplementation can result in greater muscle mass have not been conducted.

Another caveat offered by several researchers is that almost all of the positive creatine effects so far noted have been achieved in laboratory tests of elite athletes and were observed only in the sort of maximal intermittent exercise that non-athletes can rarely achieve. No benefit for any aerobic activity has been demonstrated.

In addition, safety data are lacking and are urgently needed, especially for long-term use of creatine and for use among the pediatric population (including adolescents) and among those in poor health. There are some reports that long-term use of creatine may be nephrotoxic. This needs further investigation before long-term creatine supplementation can be recommended under any circumstance.

Possible additional uses for creatine have been suggested by preliminary work. There is some evidence of creatine synthesis in the retina, and supplementation with 1.5 grams of creatine daily for a year has been reported to bring improvement in genetic gyrate atrophy—not in the blindness that results from this condition but in the skeletal muscle abnormalities that also characterize it. Giving 5 grams of creatine four times a day for a period of five days has similarly been reported to improve skeletal muscle function in some with congestive heart failure. The effect was small.

In a mouse model of amyotrophic lateral sclerosis (ALS), supplemental creatine significantly prolonged survival. Improvement was seen on motor-performance tests, and there was histologic evidence of neuron protection associated with creatine supplementation. Because creatine protected neurons in the substantia nigra, there is speculation that the supplement could also have positive effects in Parkinson's disease.

These researchers have suggested that creatine may exert the favorable results seen in the mouse model through an intracellular energy-buffering effect that may help prevent the sort of mitochondrial dysfunction that they postulate plays a role in neuronal cell death. More research is needed.

Another recent, preliminary report asserts a positive role for supplemental creatine in the treatment of muscular dystrophy and some other neuromuscular disorders. This study tested 10 grams of creatine daily for five days, followed by 5 grams daily for an additional 5 to7 days, against placebo. Increases were noted in handgrip, ankle and knee strength among those taking creatine. Again, more research is needed.

CONTRAINDICATIONS, PRECAUTIONS, ADVERSE REACTIONS

CONTRAINDICATIONS

Creatine is contraindicated in those with renal failure and renal disorders such as nephrotic syndrome.

PRECAUTIONS

Creatine supplements should be avoided by children, adolescents, pregnant women, nursing mothers and anyone at risk for renal disorders such as diabetics. Those taking creatine should have serum creatinine levels monitored.

ADVERSE REACTIONS

The deaths of three American college wrestlers had been linked to the use of creatine supplements. However, results of post mortem tests led to the conclusion that the deaths were caused by severe dehydration and renal failure, and were not due to creatine. Apparently, the wrestlers were trying to lose enough weight through perspiration to allow them to compete in lower-weight classes. Typical adverse effects are gastrointestinal and include nausea, diarrhea and indigestion. Also common are muscle cramping and strains. Weight gain may occur from water retention. During a five day loading period, weight gains of 1.1 to 3.5 pounds have been reported. There are reports of elevated serum creatinine, a metabolite of creatine and a marker of kidney function, in some who take creatine and have normal renal function. This is reversible upon discontinuation of creatine.

Anecdotal reports of adverse events to FDA have included rash, dyspnea, vomiting, diarrhea, nervousness, anxiety, migraine, fatigue, polymyositis, myopathy, seizures and atrial fibrillation.

INTERACTIONS

There are as yet no known drug, nutritional supplement or herb interactions. Caffeine (in coffee, tea and caffeinated beverages) appears to interfere with any beneficial effects of creatine supplementation.

DOSAGE AND ADMINISTRATION

The typical form of creatine available is a creatine monohydrate powder.

The dosing for those who use creatine to attempt to improve performance in brief, high-intensity activities, is a loading dose of 20 grams or 0.3 grams per kilogram in divided doses four times a day for two to five days, followed by a maintenance dose of no more than 2 grams daily or 0.03 grams per kilogram. Those who use creatine supplements should take them with adequate water, six to eight glasses per day.

HOW SUPPLIED

Capsules — 700 mg, 725 mg, 750 mg, 1200 mg
Effervescent Tablets — 5 gm
Effervescent Powder — 27 gm/packet
Powder — 5 gm/tsp
Wafers — 1000 mg

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Curcuminoids

TRADE NAMES

Curcumin is available as turmeric generically from numerous manufacturers. Branded products include Turmeric Special Formula (Solaray) and Turmeric Power (Nature's Herbs).

DESCRIPTION

Curcuminoids are polyphenolic pigments found in the spice turmeric. The term turmeric is used both for the plant *Curcuma longa* L. and the spice derived from the rhizomes of the plant. The major curcuminoids are curcumin, demethoxycurcumin and bisdemethoxycurcumin. These substances comprise 3 to 6% of *Curcuma longa*. Curcumin

TOUGH TOPICS IN SPORTS MEDICINE 1047-9651/00 $15.00 + .00

PERFORMANCE-ENHANCING SUPPLEMENTS

Matthew A. Pecci, MD, and John A. Lombardo, MD

The world of college and professional athletics is highly competitive. Athletes constantly strive to be bigger, faster, and stronger to achieve, as well as keep pace with, their competition. This is the mentality that initially fostered the use of anabolic steroids as ergogenic agents in the early 1950s. Use of other agents soon followed, including stimulants and growth hormone. Athletes continue to use these agents to gain what they consider a competitive edge over their opponents. With the ban of these agents, however, by most sports governing bodies, and because of their numerous, oftentimes dangerous side effects, demand has arisen for legal, safer alternatives. It is within this framework that the supplement market has grown and prospered.

The nutritional supplement industry generated $3.3 billion dollars in revenue in 1990,[4] and as the demand for supplements has increased, this figure has continued to rise. The use of supplements is not limited to athletes; today many people view supplements as a quick and easy way to change their appearance and look bigger or thinner. The danger is that the supplement industry is virtually unregulated by the Food and Drug Administration (FDA). Often, performance claims and side effect profiles of these agents are speculative, based on theory rather than hard evidence. Two of the agents that have received most of the publicity in the past several years are creatine and androstenedione. Claims about the ergogenic effects of these agents are numerous, and studies are ongoing to try to validate or refute some of these claims. Despite the deluge of information and popular press about these agents, it is the responsibility of physicians, trainers, and coaches to be aware of and be able to counsel athletes and patients on the facts.

From the Department of Family Medicine, Boston Medical Center, Boston, Massachusetts, USA
(MAP); and the Ohio State University Sports Medicine Center, Ohio State University,
Columbus, Ohio (JAL)

PHYSICAL MEDICINE AND REHABILITATION CLINICS OF NORTH AMERICA

VOLUME 11 • NUMBER 4 • NOVEMBER 2000

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CREATINE

Creatine has been in widespread use since the early 1990s. Today its use is prevalent in college athletes, and it is often routinely distributed by college athletic departments. Also, the use of creatine has filtered down to the high school level athlete. Nearly every high school footballer who has aspirations of playing college football has thought about trying or has tried creatine, in hopes of getting bigger and stronger. The danger with these practices is that currently one does not know the long-term side effects of creatine use, and the effect of creatine on children or adolescents is not known. In addition, there are many conflicting studies in the literature, and the data are still inconclusive as to whether creatine is even beneficial as an ergogenic agent.

History

Creatine was first recognized as a component of meat in 1832 by a French scientist, Chevreul. In 1847, Lieberg performed animal studies confirming that creatine was contained in muscle tissue, and its accumulation was enhanced by muscle work.[26] Shortly after the discovery of creatine, the substance currently known as creatinine was discovered in urine. The amount of creatinine excreted seemed to be related to muscle mass; it was theorized that creatinine was derived from the breakdown of muscle stores of creatine.[6]

Further studies and observations from the early 1900s demonstrated that not all ingested creatine could be recovered in the urine. This information suggested that some of the ingested creatine remained in the body. Again, animal studies later confirmed that much of the ingested creatine was actually retained in the muscles.[6] This early work proved that creatine was in fact a component of muscle tissue, but its function in the muscle was still unknown. The discovery of phosphocreatine by Fiske and Subbarow in 1927, however, led to speculation that creatine was important in skeletal muscle energy and metabolism.[37]

It was not until 1967 that the first human data on creatine usage began to be accumulated by Hultman.[23] He studied creatine's role in muscle metabolism using muscle biopsy techniques to study adenosine triphosphate (ATP) and phosphocreatine breakdown and resynthesis in exercising humans. His work demonstrated that creatine played a role in muscle ATP synthesis and muscle energy in humans. Despite this knowledge, it took more than 10 years before creatine was suggested as a possible ergogenic agent. The first suggestion of its ergogenic potential was by Sipila[46] in 1981. In his study, he was using creatine to treat gyrate atrophy, and noted that his subjects reported a subjective increase in strength. The hallmark study that is associated with the surge in human use of creatine as an ergogenic agent, however, was performed by Harris[20] in 1992. He demonstrated that oral creatine supplementation in humans resulted in an increase in skeletal muscle creatine concentration. Despite the fact that there was no evidence at the time to suggest that creatine enhanced strength or athletic performance, because of its known role in skeletal muscle energy and metabolism, and now knowledge that supplementation increased creatine muscle content in humans, it was quickly marketed as an ergogenic agent. Since then, creatine usage has dramatically increased, as have the studies on its role as an ergogenic agent.

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Biochemistry

Creatine is a nitrogenous compound synthesized in vivo from the amino acids glycine, arginine, and methionine. The two enzymes involved in the synthesis of creatine are transamidinase, which transfers the amidine group from arginine to glycine, and methyltransferase, which adds a methyl group from S-adenosylmethionine.[6] The synthesis of creatine occurs in the liver, pancreas, and kidney at a rate of about 1 to 2 grams per day. Creatine then must be transported to the muscle through the bloodstream. It is taken up by the muscle by a sodium-dependent transport system that has been shown to be up-regulated by insulin and down-regulated by exogenous creatine.[16, 36]

In addition to endogenous synthesis, approximately 1 to 2 grams per day of creatine is obtained exogenously through dietary consumption. The main sources of creatine are fish and meat. Lesser sources include milk and cranberries.[6] Creatine is excreted by the kidneys at a rate of approximately 1 to 2 grams per day, after irreversible conversion to creatinine.[55] It may be excreted, however, in an unconverted form with excessive dietary intake.

Skeletal muscle contains approximately 95% of the creatine stores in the body. Other areas where creatine can be found include the heart, testes, brain, and retina.[56] Creatine exists in the tissues in two forms: creatine and phosphocreatine. The total creatine concentration in muscle may range from 90 to 160 mmol/kg,[17] and approximately two thirds of this is in the form of phosphocreatine. Vegetarians generally have lower concentrations[14] because of their lower dietary intake. Also, stores of creatine tend to vary depending on muscle fiber type, with type II fibers having a higher level of phosphocreatine than type I fibers.[48, 49]

The main role of phosphocreatine is to provide energy to the exercising muscle in the form of ATP. It accomplishes this through the donation of a high-energy phosphate group, leading to the rephosphorylation of adenosine diphosphate (ADP) into ATP. This process occurs anaerobically and is catalyzed by the enzyme creatine kinase. Phosphocreatine is the rate limiting factor in this equation, and stores of phosphocreatine generally are depleted after 10 to 20 seconds of exercise.[24] After this short period of time, aerobic metabolism takes over and glucose and fatty acids primarily are used to regenerate ATP.

As mentioned previously, creatine use as an ergogenic agent began in the early 1990s after a study by Harris[20] found that oral creatine given as a supplement caused a rise in total creatine content in muscle. In addition, the study showed that 20% of the increase in total muscle creatine was caused by an increase in phosphocreatine. This occurs because creatine and phosphocreatine exist in equilibrium at rest,[17] and increasing the levels of one should theoretically increase the other. The basis for creatine use as an ergogenic agent is this rise in phosphocreatine. As previously stated, phosphocreatine is the rate limiting step in conversion of ADP to ATP. So, it is proposed that by supplying the muscle with more phosphocreatine in effect more ATP can be generated, and the muscle has more energy for short burst anaerobic activity.

Supplement Dosing

Creatine supplied orally is taken up by skeletal muscle.[20] Muscle does not, however, continuously take up all the creatine that is taken in orally. Numerous studies have documented that muscle has a creatine saturation limit.[17, 20, 25] This limit is approximately 150 mmol/kg to 160 mmol/kg.[31] Once this limit is

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reached, urinary excretion of creatine begins to equal oral intake.[20] Several different regimens of oral supplementation have been studied. Creatine can be supplied either with an initial loading dose of 20 to 25 grams daily (0.3 g/kg) for 5 days, then at a maintenance level of 2 grams daily (0.03 g/kg). Alternatively, creatine may be supplemented with no loading phase at 3 grams daily.[25] Both of these regimens effectively saturate the muscle to its limit; however, with no loading phase it takes approximately 28 days to achieve muscle saturation, whereas saturation can be achieved quickly after 5 days of loading.[25] Once this saturation limit is achieved, it can be maintained with the lower dosage of 2 grams daily. There are no data to suggest that one regimen is safer than the other. Additionally, it has been shown that ingesting carbohydrates, such as a sports drink, with creatine augments creatine uptake into the muscle.[16] This is believed to occur because of insulin up-regulation of the creatine receptor.

When creatine supplementation is discontinued, it takes approximately 28 days for levels in the muscle to return to baseline.[25, 51] Renal excretion of creatinine and creatine also returns to baseline after 28 days.[51]

Ergogenic Potential

Does creatine have any beneficial effects on athletic performance? Certainly, there are numerous anecdotal reports and information in the popular press touting the benefits of creatine supplementation. There also have been numerous studies within the last 10 years exploring creatine's effect on physical performance. These studies have been analyzed extensively in several excellent review articles.[6, 30, 31]

The performance claim that seems to be most often associated with creatine use is that it enhances sprint performance. This seems to make logical sense given that creatine supplementation increases muscle phosphocreatine, which is the main source of ATP during short burst, high intensity activities. This claim, however, is an overgeneralization given the data that exist. Most studies that have looked at a maximum effort single sprint on a cycle ergometer have shown no benefit on performance with creatine supplementation.[12, 13, 40, 47] Additionally, studies exploring the potential benefit of creatine supplementation on running and swimming sprints also have shown no benefit with supplementation.[9, 19, 29, 39, 44, 50] The only consistent benefit shown in regards to sprinting have been seen in several studies looking at repetitive bouts of sprinting on the cycle ergometer. Several of these studies have demonstrated a benefit during the initial trial with creatine supplementation.[8, 13, 15, 35, 42] Overall, the results seem to be inconclusive as to whether or not creatine supplementation has any ergogenic effect on a single sprint. So far most of the data suggest it does not, especially in running and swimming sprints.

The other major ergogenic claim of creatine use is that it makes one bigger and stronger. Again, biochemically this may make sense given the fact that supplementation increases phosphocreatine. Theoretically there would be more ATP available to the muscle, and potentially the muscle can contract more in a given time period. By contracting more, it would be expected that there is an increased stimulus for muscle growth. Furthermore, it has been proposed that creatine may directly enhance protein synthesis, and this has been demonstrated in vitro.[27, 28] In regards to the data available, only a small number of studies exist, and they seem to demonstrate an increase in weight-lifting repetitions (bench press, leg press) after creatine supplementation.[15, 35, 51, 53] Two of the studies also show an increase in one repetition maximum.[15, 51] Numerous studies

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also have documented an increase in total body mass in subjects supplementing with creatine.[13, 15, 35, 39, 47, 51, 53] This increase in mass generally is observed shortly after the creatine load, which would be too quick for a significant amount of muscle protein synthesis to occur and account for this increase. It is believed that this initial gain in weight is in fact caused by water retention.[31] It has been demonstrated that creatine loading does in fact cause an initial decrease in urinary output.[25] Longer term gains, however, may be caused by increases in muscle protein synthesis and lean body mass,[15, 35, 51] although this has not been proven definitively as of yet. Overall, the current knowledge suggests that creatine supplementation does in fact enhance strength and increase body mass, which long term may be the result of increase in muscle size.

Other areas of performance where creatine use has been studied is in multiple bouts of high intensity activity. Thus far the preponderance of the evidence, which mostly looks at cycle ergometry, suggests that creatine may enhance performance in successive bouts.[5, 8, 13, 15, 35, 42] It has been proposed that this is because of an increase in phosphocreatine resynthesis with supplementation, thereby making more phosphocreatine available for successive bouts. One study available seems to confirm this hypothesis,[18] whereas another refutes it.[52] The exact mechanism of this ergogenic benefit is still unknown. This may be an important ergogenic effect, because many sports require repetitive, short bursts of high intensity activity (i.e., football, basketball, hockey, tennis). It must be emphasized that this effect only has been observed in the laboratory setting, and there is as of yet no data of an ergogenic benefit in actual athletic competition.

Side Effects

It is important that one considers the side-effect profile of any ergogenic agent, particularly one that has achieved such widespread use in such a short period of time. As stated previously, the FDA does not regulate the supplement industry; there does not need to be extensive data on potential side effects of a product before it is marketed to the general public. Currently, there are no studies demonstrating any serious side effects of creatine supplementation. There exist numerous case reports, however, potentially linking creatine use to some adverse effects, but so far a cause and effect relationship has not been established.

The organ that has received the most attention as a potential site of adverse side effects of creatine supplementation is the kidney. There have been two case reports of renal damage potentially related to creatine use published in the literature in the last several years.[34, 43] In one case, however, the subject had a baseline renal abnormality, and in the other case the subject ingested higher than the recommended dose of creatine (20 grams daily for 4 weeks). Creatine is excreted solely by the kidneys, and supplementation has been shown to increase urinary excretion of creatine[41, 51] and creatinine.[25, 45, 51] These urinary levels return to baseline approximately 28 days after discontinuing supplementation,[25, 51] and as of yet there are no data to suggest that these increased levels have any significant effect on renal function. Also, there have been no documented changes in glomerular filtration rate (GFR) or protein excretion with creatine supplementation.[41] It must be emphasized that currently there is no information on the renal effects of long-term creatine supplementation. Presently, the only guidelines that exist for creatine use, in regards to the renal system, are that it should not be used in anyone with pre-existing renal disease,

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and those with a potential for renal dysfunction (elderly, hypertensive, diabetic) should have their renal function carefully monitored[48] when using creatine.

Another potential side effect often linked to creatine usage is dehydration. Although there seems to be no increased incidence of dehydration in any of the studies that exist on creatine use, certainly anecdotal evidence of this phenomenon is quite common. Most manufacturers of creatine even emphasize the importance of proper hydration when taking this supplement. As stated previously creatine actually decreases urinary output acutely.[25] This theoretical risk of dehydration is not linked to increased fluid output. Rather, it is thought that some of the intravascular volume is redistributed into the muscle,[30] leading to a relative state of dehydration. If an athlete taking creatine then exercises vigorously and additionally loses fluid through sweating, he or she may be at an increased risk of becoming clinically dehydrated. This increased risk of dehydration also may make one more prone to muscle cramping, which has been associated with creatine usage. Again, no studies have proven this association, but anecdotal evidence abounds. It also has been hypothesized that muscle cramping may be caused by fluid shifts into the muscle, causing a compartment syndrome type effect.[32] Currently, the recommendation has been to increase fluid intake while taking creatine, especially when combined with vigorous exercise. Also, during times when an athlete may be particularly prone to dehydration, such as with workouts during the summer months or during two-a-day football practices, it is suggested that he or she discontinues creatine use.

Other potential areas of concern include creatine's effect on the liver. No long-term studies exist; however, short-term studies have not demonstrated any significant elevation in liver enzymes.[3, 35] Also, no data exist on creatine use in children and adolescents, despite the fact that this is one population where creatine use has skyrocketed. Because of this lack of data, the current stand has been to discourage creatine use in children or adolescents, emphasizing instead a maximization of their training regimen to promote strength or size. Finally, it is also important to remember that creatine exists in many other tissues besides the muscle, and one does not have any evidence on the effect of creatine supplementation in these areas. Certainly further evidence needs to be gathered before one can truly promote creatine as a safe ergogenic agent.

ANDROSTENEDIONE

Androstenedione (Andro) is an endogenously produced steroid that has emerged on the supplement market over the past few years as a natural anabolic steroid. Aided by the revelation that a star professional baseball player regularly used it as part of his training regimen, the popularity of andro has grown rapidly over the past year. Andro belongs to a class of agents the supplement market calls *prohormones* because of the fact that they are precursors to testosterone. Andro is marketed as a legal anabolic steroid, with the theoretical ability to increase protein synthesis and muscle size similar to its close relatives, the illegal synthetic anabolic steroids. Also, because it is endogenously produced, it is not believed to have some of the well-documented side effects of the synthetic oral steroids, mainly liver abnormalities and tumors. Most of the claims about andro, however, currently are unsubstantiated. Studies are just beginning to emerge looking at the potential anabolic activity of this agent. At the time this article was being written, only one prospective randomized trial on androstenedione supplementation existed in the literature[33] (in JAMA), but more have since been published. Also, as with many of the supple-

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ments, the side effects of androstenedione use are still unclear. Certainly more data need to be gained on the potential efficacy and safety of this agent before recommending it for use as an ergogenic supplement.

Biochemistry

Androstenedione is produced in the body by the adrenal glands, and to a lesser extent by the gonads. It also is produced by some plants, most notably wild yams. The common precursor to androstenedione, as in all steroid hormones, is cholesterol (Fig. 1). The basis for its structure is the 19-membered steroid ring. It can be formed in one of two pathways, either from dehydroepiandrosterone (DHEA) by the action of the enzyme 3beta-OH steroid dehydrogenase-isomerase, or from 17-OH progesterone by the action of the enzyme $C_{17\text{-}20}$ lase.[11] The androstendione produced then may undergo reversible conversion to testosterone by the enzyme 17beta-OH steroid dehydrogenase, or it may be aromatized to estrone. This conversion to testosterone can occur in many sites in the body, because the enzyme 17beta-OH steroid dehydrogenase is found in most body tissues.[10]

Androstenedione is an androgenic, anabolic steroid, but it is unclear whether it can exert these effects itself, or whether its actions first necessitate its conversion to testosterone.[11] The so-called androgenic effects are those that are related to the reproductive function in males. These include male reproductive tract differentiation in utero, maturation of external genitalia at puberty, development of male secondary sex characteristics, and regulation of sperm production.[11] The anabolic effect is the action of inducing protein synthesis. It is this effect, particularly protein synthesis in muscle tissue, that is most attractive to the athlete. Testosterone, and possibly its precursor hormones, exert their effect by binding to specific receptors in cell cytoplasm. These receptor complexes are

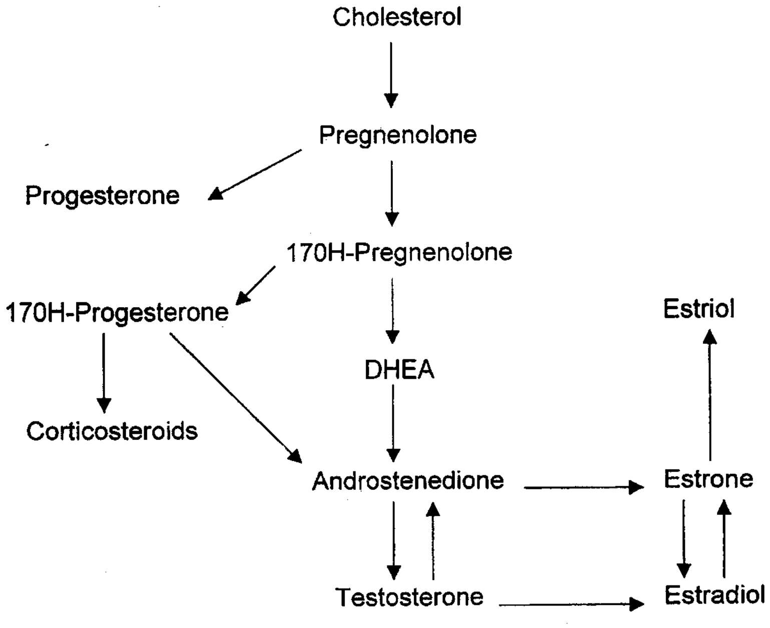

Figure 1. Androstenedione production.

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then transported to the cell nucleus, where they can activate certain genes responsible for protein synthesis.[54] The type of protein synthesized seems to depend on the specific cell where the hormone is exerting its action.

The steroid hormones, including androstenedione, are metabolized mainly in the liver. Here they undergo a series of reduction reactions, and are ultimately conjugated with glucuronide or sulfate moieties. This conjugation increases their water solubility and allows them to be excreted in the urine.[11]

Ergogenic Effects

As stated previously, it is as of yet unclear whether androstenedione itself is anabolic. Most experts believe that androstenedione is at best weakly anabolic; however, most of the effect of the endogenously produced hormone likely results from conversion to the more potent testosterone. One can assume for supplementation with andro to have any significant anabolic effect, it either needs to be supplied in very large doses, or it needs to be converted to testosterone. Many studies have already documented the anabolic effect of supplemented testosterone on strength and lean body mass.[1, 7] There are very little data available, however, documenting whether in fact orally administered androstenedione is converted to testosterone in any appreciable amounts. Several studies from the early 1960s looking at very small numbers of women have actually shown a measurable increase in testosterone after oral administration of 100 mg/day of androstenedione, as well as after intravenous infusion.[22, 38] The first recent study on oral androstenedione administration, however, failed to demonstrate any increase in serum testosterone with andro supplementation.[33] Contrary to the earlier study, this study was performed on male subjects and used a higher dose of andro of 300 mg/day. There are several possible explanations for the discrepancy. First, it is possible that males may require a much higher dose of supplemented andro to exhibit any significant conversion to testosterone. In fact, even though the recommended daily dose of androstenedione is 100 to 300 mg/day, many users actually ingest two and three times this dose. Also, as postulated in the JAMA study, there was a significant increase in estrogen concentration with andro supplementation, suggesting perhaps that any testosterone made may have been rapidly aromatized. In any case, the data from this study do not seem to suggest a significant conversion of androstenedione to testosterone in males at a dose of 300 mg/day. Several articles have been published since this article was originally written, however, that have shown an increase in serum testosterone concentration after oral administration of 300 mg of androstenedione. The data, therefore, is currently conflicting as to whether serum testosterone increases after ingestion of androstenedione at 300 mg/day.

The study in JAMA ultimately did not demonstrate any significant gains in strength or size in those taking androstenedione for 8 weeks versus those taking placebo. Again this is the only study currently available exploring the potential anabolic effects of andro supplementation, and more are on the way. There are certainly numerous anecdotal reports, however, particularly from the body building community, making claims about the anabolic effects of this agent. A final point that must be made is that the JAMA study population were subjects who were not engaged in a regular weight training regimen before entering the study. In inexperienced weight lifters, the initial gains in strength are believed to be caused by neuromuscular adaptation. This phenomenon, as demonstrated in earlier studies on anabolic steroids, does not seem to be enhanced by the intake of ergogenic agents. One would not expect to see any effect of ergogenic agents in this particular population. In the studies on the ergogenic benefits of

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synthetic anabolic steroids, these agents only seemed to demonstrate a significant increase in size and strength in those athletes who were already involved in a regular resistance training program. With regular, high intensity weight training, an athlete is forced into a relative catabolic state, primarily by the release of glucocorticoids. In this situation the anabolic steroids reverse this catabolic state, causing a significant increase in strength gains and lean body mass.[21] If one is not in this catabolic state, anabolic steroids have shown no or little effect. So, it is possible that androstenedione may exert its effect in a similar manner, and to be adequately studied, the study population must be experienced weight lifters. As demonstrated by this discussion, so far the data on androstenedione and its ergogenic effects are scarce, and more information is needed before any definitive conclusions can be made.

Side Effects

The data on the side effects of androstenedione supplementation, similar to that of creatine, is minimal. It seems from the limited knowledge that orally administered androstenedione, to some extent, aromatizes to estrogen.[33] One can assume that, like synthetic anabolic steroids, andro may have feminizing side effects (e.g., gynecomastia) and the development of a high-pitched voice in the male. Also, if andro has anabolic effects, it likely has androgenic effects as well. It has the potential to cause deepening of the voice, clitoromegaly, and hirsuitism in females, and acne and male pattern baldness in both sexes. So far, it has not been shown to inhibit production of luteinizing hormone or follicle-stimulating hormone,[33] which could cause testicular atrophy and azoospermia.

It is currently unknown whether androstenedione produces some of the other side effects often attributed to synthetic anabolic steroids. Theoretically it should not cause any of the serious hepatic side effects seen with the use of orally administered synthetic anabolic steroids, because this effect is believed to be potentiated by 17 alpha alkylation,[21] which androstenedione does not have. Thus far, there have been no documented liver enzyme (LFT) abnormalities with androstenedione supplementation.[33] Synthetic anabolic steroid usage also has been shown to cause lipid abnormalities, specifically increased low-density lipoproteins (LDL) and decreased high-density lipoproteins (HDL).[2] A significant decrease in HDL with androstenedione supplementation has been documented, but no change in LDL or triglycerides.[33] Finally, there are currently no documented cases of the more serious complications of anabolic steroid use with androstenedione, namely prostate, kidney, or pancreatic tumors. Once again it must be emphasized, however, that the data are minimal, and more information needs to be uncovered.

SUMMARY

Supplements that are marketed as ergogenic aids have achieved widespread use in the United States. In image-conscious society, these agents are not only being consumed by athletes, but also by those looking for a quick fix to enhance their appearance. Many assume that the performance claims made by the manufacturers are based on actual data, and that these agents must be safe because they are sold to the general public. Unfortunately, in most cases these assumptions are false.

Creatine has become very popular, particularly among college and high school athletes. Studies within the last 5 years have shown that creatine does seem to have certain ergogenic benefits in a laboratory setting. It is not currently known whether these benefits actually can be transferred to the playing field.

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Although creatine has not consistently been shown to cause any major side effects, there is some question regarding creatine's effect on the kidneys, particularly with long-term use. Also, the safety of supplementation in children and adolescents has not been examined at all; its use in this population should be discouraged until there are more data.

Androstenedione is an agent that has received a large amount of popular press in the last year, and this has led to an surge in its usage. It is believed to exert its ergogenic effects through conversion to testosterone. But what limited data are available suggest that at the recommended dosage, it does not cause any measurable change in testosterone levels, or provide any ergogenic benefit in inexperienced weight lifters. Also, it has yet to be determined whether androstenedione causes any of the side effects often attributed to use of the illegal anabolic steroids. Its mechanism of action suggests it has the potential to cause many of these negative effects. Studies are just beginning to appear in the literature, and certainly more data need to be gathered before androstenedione supplementation can be recommended for use as an ergogenic aid.

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Address reprint requests to

Matthew A. Pecci, MD
Ohio State University Sports Medicine Center
2050 Kenny Road
Columbus, OH 43221

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Food and Chemical Toxicology 44 (2006) 2086–2091

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Formation of mutagenic heterocyclic aromatic amines in fried pork from Duroc and Landrace pigs upon feed supplementation with creatine monohydrate

Wolfgang Pfau [a,*], Katja Rosenvold [b], Jette F. Young [c]

[a] *Umweltmedizin Hamburg e.V. and Institute of Toxicology, Hamburg University, D-22527 Hamburg, Germany*
[b] *Danish Meat Research Institute, Department of Pork and Beef Quality, DK-4000 Roskilde, Denmark*
[c] *Danish Institute of Agricultural Sciences, Department of Food Science, Research Centre Foulum, DK-8830 Tjele, Denmark*

Received 15 December 2005; accepted 10 July 2006

Abstract

Heterocyclic aromatic amines (HAA) have been shown to induce tumours at various organ sites in experimental animal studies and high levels of dietary intake of HAA have been associated with increased cancer risk in humans. These HAA are formed in meat upon heating from precursors such as amino acids, reducing sugars and creatine or creatinine.

Groups of ten Duroc and ten Landrace pigs received feed supplemented with creatine monohydrate (CMH) for five days prior to slaughter at dose levels of 12.5, 25 and 50 g per animal per day. Ten control animals of each breed received the non-supplemented feed. Meat from Duroc pigs had been shown to respond to CMH supplementation with regard to waterholding capacity, juiciness, post slaughter pH and colour parameters, meat from Landrace pigs was unaffected.

Indeed, while creatine phosphate levels in meat from Duroc pigs increased in a dose-dependent manner with CMH supplementation, no effect was observed in meat from Landrace pigs.

Meat slices from longissimus dorsi were fried and considerable mutagenic activity was detected in meat extracts in Salmonella typhimurium YG1019 in the presence of rat-liver homogenate. However, no effect of breed or CMH supplementation was observed in fried pork on the formation of HAA determined as mutagenic activity. It may be concluded that feed supplementation with CMH at levels up to 50 g per day for five days prior to slaughter does not increase the level of heterocyclic aromatic amines detected as mutagenic activity formed upon frying of pork.

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Keywords: Mutagenic; Creatine; Pork

1. Introduction

Contaminations or residues in animal feed but also feed additives and supplements may influence the quality and safety of meat. At the other end, heating of food may introduce toxic compounds such as heterocyclic aromatic amines (HAA) or acrylamide (Jägerstad and Skog, 2005).

HAA have been shown to induce cancer in experimental animals. Because of their occurrence in cooked meat products, these HAA may contribute to common forms of human cancer including colorectal, breast, and prostate tumours that are associated with frequent consumption of diets high in meats (Knize and Felton, 2005).

HAA are formed when proteinous matter is heated. Among these, the amino-imidazo azarenes (AIA) have

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Abbreviations: AIA, amino-imidazo azarenes; HAA, heterocyclic aromatic amines; LD, logissimus dorsi; CMH, creatine monohydrate; CP, creatine phosphate; WHC, water holding capacity.

[*] Corresponding author. Present address: GAB-Consulting GmbH, D-21769 Lamstedt, Hinter den Höfen 24, Germany. Tel.: +49 40 67046759; fax: +49 4773 8889 20.

E-mail address: wolfgang.pfau@GAB-consult.de (W. Pfau).

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doi:10.1016/j.fct.2006.07.011

been demonstrated to be most potent in microbial mutagenicity assays and in oncogenicity studies. These AIA are found in grilled, fried and cooked meat and fish. The levels vary in the low ppb range and depend mainly on heating time and temperature but are also influenced by water content, proportion of amino acids and type of meat (Skog et al., 1998; Knize and Felton, 2005). Thus, human exposure may add up to several hundred nanograms per day per kg body weight. PhIP and MeIQx are the most prominent AIA with MeIQx accounting for more than 90% of the mutagenic activity found in meat fried moderately under household conditions (Jägerstad and Skog, 2005; Brockstedt, 1997).

Creatinine has been identified as a precursor of AIA formation (Jägerstad et al., 1984) and fortification of meat juice or model systems with creatine or creatinine resulted in increased formation of mutagenic AIA (Övervik et al., 1989). Therefore, animal feed supplementation with creatine monohydrate may result in increased levels of creatine in the meat and, thus, an increased human exposure towards AIA.

Creatine is being used as a legal ergogenic aid and has been shown to enhance athletic performance when taken at the recommended dosing regimen and as supplementation of an exercising program (Volek and Rawson, 2004). In human studies, supplementary creatine has been shown to increase total muscle creatine (Balsom et al., 1995), of which approximately two-thirds is creatine phosphate (Balsom et al., 1994; Casey et al., 1996).

Creatine as a feed supplementation has been proposed for pigs in order to improve meat quality, delaying lactate formation in the muscle and consequently postpone the pH decline *post mortem*. Berg and Allee (2001) proposed a hypothesis of increased water holding capacity (WHC) in meat from pigs supplemented with creatine. Alteration in the water mobility has been indicated in several studies by tendencies to greater myofiber hydration (Berg and Allee, 2001), reduced cooking loss (Maddock et al., 2002) and increased WHC. Furthermore, differences in muscle composition between breeds (Ruusunen and Puolanne, 1997) may have an impact on the overall metabolism in the muscle. Comparing two breeds, Duroc and Landrace pigs, it has been shown that meat from *Longissimus dorsi* (LD) of Duroc pigs had a higher WHC and pH post slaughter, lower colour determinants and was more juicy compared to that of Landrace pigs (Young et al., 2005). Dietary supplementation with CMH increased the body weight gain of both breeds and redness of the meat was reduced. However, only meat from Duroc pigs had higher pH-values shortly after slaughter and higher WHC, but juiciness was reduced upon CMH supplementation (Young et al., 2005).

The objectives of this study were to investigate the influence of dietary CMH supplementation in Duroc and Landrace pigs on the creatine content in the meat and the formation of mutagenic AIA upon frying.

2. Materials and methods

2.1. Animals and management

The experiment comprised a total of 80 female pigs, 40 Duroc and 40 Landrace of pure breeds obtained from Breeding centre Kollund, Herning, Denmark. The pigs were transported to the stables at the Danish Institute of Agricultural Sciences, Research Centre Foulum, 13 d before slaughter and penned in pairs. Animals were gradually being introduced to the basic feed over the first seven days. Five days before slaughter pigs were individually penned and respective breeds were distributed on the experimental feeds ($n = 10$) consisting of the basic feed supplemented with 0, 12.5, 25 or 50 g creatine monohydrate (Micronized creapure, Degussa Food Ingredients, D-85354 Freising, Germany) (CMH)/d. For the five days pigs were fed 2×1375 g basic feed or feed mixed with CMH at two fixed time points during the day related to the time of slaughter (last feed 18 h before slaughter). Remaining feed was registered after every feed, and the body weight was registered before and after the experimental feeding period i.e. in the morning five days before slaughter and before transport to the slaughter plant.

2.2. Determination of creatine phosphate

Determination of creatine phosphate (CP) was performed by HPLC as described by Henckel et al. (2002) except increasing the sample size and extraction volumes five times.

2.3. Meat preparation

Samples for determination of HAA were cut 24 h post mortem from *longissimus dorsi* (LD). The samples were vacuum packed, stored at 4 °C for 24 h and kept at −20 °C until cooking. Prior to cooking the samples were thawed at 4 °C for 24 h. One cm thick slices were fried on a frying plate at 200 °C for 6 min on both sides and were edible. Following frying the samples were cooled, vacuum packed and stored at −20 °C until further analysis.

2.4. Sample preparation

Aliquots of fried meat (1 g) were homogenized with an ultra turrax and subjected to a tandem column extraction procedure according to Gross (1990) with variations as described by Messner and Murkovic (2004) using diatomeous earth and Oasis SPX columns with ethyl acetate as eluent. Extracts were dried *in vacuo* and redissolved in DMSO.

2.5. Bacterial mutagenicity

Extracts from fried meat were assessed for mutagenicity towards the frame shift detecting strain *Salmonella typhimurium* YG1019 (a generous gift from P.D. Josephy, University of Guelph, Ont., Canada). Pre-incubation assays were performed in the presence of Aroclor 1254-induced rat-liver S9 prepared from male Wistar rats with a 30 min pre-incubation at 37 °C (Pfau et al., 1999). The final S9 mix (30% v/v) containing 33 mM KCl, 8 mM $MgCl_2$, 5 mM glucose-6-phosphate, 3.3 mM $NADP^+$, 100 mM phosphate buffer, pH 7.4. Revertant colonies per plate were scored following 72 h incubation at 37 °C.

2.6. Data analysis

The statistical analysis was carried out with the Statistical Analysis System version 8.2 (SAS Institute, Cary, NC, USA) using the MIXED procedure. The model included the fixed effects of treatments (four CMH levels and two breeds) as well as their interaction, and the random effect of slaughter day. Orthogonal contrasts of control versus CMH supplementation as well as linear effects were estimated. LSMeans were calculated and considered to be significantly different if $P < 0.05$. Correlation coefficients were calculated on the residual variation following correction for fixed effects. These calculations were conducted with the manova option in the GLM procedure.

3. Results

3.1. Mutagenic activity

Pork meat slices were fried until still edible and meat extract were prepared according to published solid phase extraction procedures.

Using the pre-incubation assay with the tester strain *Salmonella typhimurium* YG 1019 in the presence of rat liver S9-mix considerable mutagenic activity was detected in all fried meat samples. Mutagenic activity ranged from 110–3014 revertants per g fried pork sample corresponding to 0.03–0.8 ng MeIQx per g fried meat.

The mutagenic activity extracted from meat from Duroc pigs was higher as compared to meat from Landrace pigs, but this difference did not reach significance (Table 1).

No dose response correlation was notable for CMH feed supplementation on mutagenicity of fried meat extracts for either race (Fig. 1). The increase in mutagenic response observed in meat from Duroc pigs at supplementation levels of 12.5 and 50 g CMH/d was not significantly different from the control (Table 2). The formation of HAA in meat from Duroc pigs was not significantly increased by CMH feeding, but when comparing the control to all of the CMH supplemented pigs there was a tendency ($P = 0.143$) towards increased mutagenicity in meat from CMH supplemented Duroc pigs. A similar effect was not found in meat from Landrace pigs.

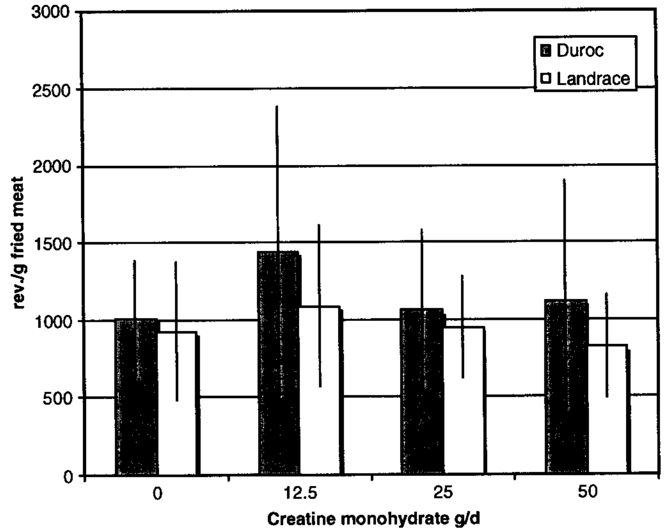

Fig. 1. Mean mutagenicity of extracts in YG 1019 shown as revertants per g fried pork derived from Duroc or Landrace pigs supplemented with CMH at 0, 12.5, 25 or 50 g per animal per day, error bars indicate standard deviation.

3.2. Creatine phosphate

Since approximately two-thirds of muscle creatine is in the form of CP (Balsom et al., 1994; Casey et al., 1996) fresh meat was analysed for CP content.

There was a tendency towards higher levels of CP in meat from Duroc pigs compared to meat from Landrace pigs. CP levels ranged from 4.2 to 23.4 µmol/g in meat from Duroc pigs and 7.0 to 18.7 µmol/g in meat from Landrace pigs.

Table 1
Effect of breed and CMH supplementation on CP and HAA contents

	Duroc	Landrace	SEM	P_{breed}	Creatine monohydrate (g/d)				SEM	P_{creatine}	$P_{\text{creatine linear}}$	$P_{\text{breedxcreatine}}$
					0	12.5	25	50				
HAA	1136	950	107	0.132	922	1252	983	1015	144	0.239	0.952	0.665
CP	13.43	12.54	0.71	0.281	11.587	12.791	13.278	14.284	0.917	0.148	**0.001**	0.413

CP: mean creatine phosphate level (µmol/g fresh meat); HAA: heterocyclic aromatic amines, mean level of revertants per g fried meat in YG1019 with metabolic activation; P_{breed}: P-value for effect of breed; P_{creatine}: P-value for the effect of creatine; $P_{\text{creatine linear}}$: P-value for a linear effect of creatine; $P_{\text{breedxcreatine}}$: P-value for interaction between breed and creatine.

Table 2
Effect of CMH supplementation to Duroc and Landrace pigs on CP and HAA contents

		Creatine monohydrate (g/d)				SEM	P_{creatine}	$P_{\text{creatine linear}}$	$P_{\text{creatine contrast}}$
		0	12.5	25	50				
Duroc	CP	11.17	12.79	14.68	15.09	1.34	0.158	**0.002**	**0.029**
	HAA	920	1415	1021	1180	226	0.341	0.670	0.143
Landrace	CP	12.00	12.79	11.88	13.47	1.12	0.664	0.184	0.600
	HAA	924	1084	945	825	148	0.622	0.442	0.860

CP: mean creatine phosphate level (µmol/g fresh meat); HAA: heterocyclic aromatic amines, mean level of revertants per g fried meat in YG1019 with metabolic activation; SEM: standard error of the mean; P_{creatine}: P-value for the effect of creatine; $P_{\text{creatine linear}}$: P-value for a linear effect of creatine; P_{contrast}: P-value for the effect of creatine (all levels) compared to the control.

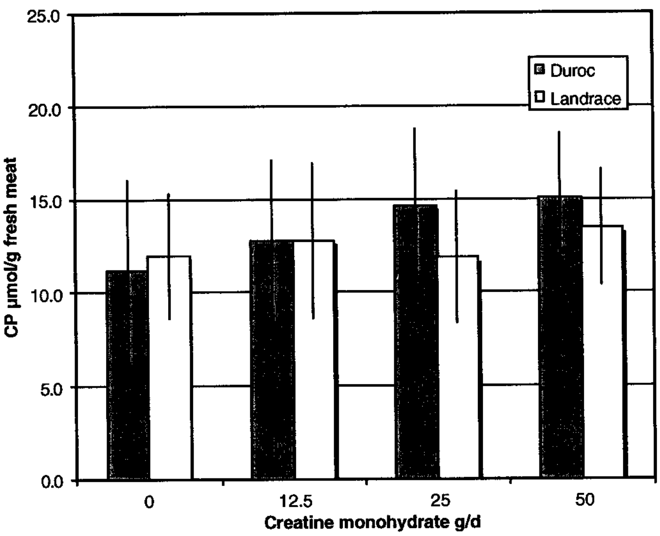

Fig. 2. Mean creatine phosphate level (µmol) per g fresh pork derived from Duroc or Landrace pigs supplemented with CMH at 0, 12.5, 25 or 50 g per animal per day, error bars indicate standard deviation.

No overall significant effect of CMH supplementation on CP levels in the meat was observed, however, the CP content increased with increasing CMH supplementation as indicated by the significant linear effect (Table 1).

Throughout the study analysis has shown that the Duroc pigs respond to the CMH feed whereas Landrace pigs responds very little or not at all. Likewise, supplementation with CMH significantly increased the CP levels in meat from Duroc pigs, whereas only 50 g CMH/d caused a slight non-significant increase in meat from Landrace pigs (Fig. 2 and Table 2).

There was no correlation between HAA levels and CP contents in the meat (Table 3). HAA was not correlated to pH of the muscle at any of the measured time points (up to 24 h) post mortem. HAA was also not correlated to the drip loss.

4. Discussion

In a comprehensive "stable to table" approach to secure food safety, it is necessary to investigate the possible effects of feed supplements on the formation of dietary carcinogens upon cooking. This is the first report investigating the effect of feed supplementation on the formation of mutagenic HAA in fried meat.

LD pork slices were pan-fried and, upon solid phase extraction using published protocols, all extracts exhibited mutagenicity in *Salmonella typhimurium* YG 1019 with metabolic activation. The observed mutagenic activity corresponded well with earlier data on mutagenicity of fried pork (Övervik et al., 1989). Also, the mutagenic response correlated well with data on the content of mutagenic HAA in moderately fried pork (Sinha et al., 1998; Skog et al., 1998; Olsson et al., 2002) taking into consideration the specific mutagenicity of these HAA in this strain of *Salmonella typhimurium* (Pfau et al., 1999). Bacteria strains such as YG 1019 carry genes for N-acetyltransferases on multicopy plasmids, which catalyse the N-acetylation of aromatic amines and the O-acetylation of hydroxylamines, the latter being formed upon metabolic activation of HAA with the aid of rat liver microsomes added to the preincubation assay. Consequently these strains are very sensitive toward HAA.

In preliminary experiments meat samples fried at different temperatures were analysed using published extraction procedures. HPLC analysis with UV-DAD detection of the extracts yielded HAA, with the number and concentrations increasing with duration of frying (Brockstedt, 1997). Observed mutagenicity was compared to calculated cumulative mutagenicity as expected from published specific mutagenic potencies of MeIQx (3800 revertants/ng) and PhIP (5 revertants/ng) (Pfau et al., 1999). Calculated cumulative mutagenicity accounted for 96.8–24.4% of the mutagenicity detected in polar meat extracts. Furthermore, the mutagenicity assay proved more sensitive and allowed detection of HAA levels corresponding to 0.03 ng/g (threefold background mutagenicity) (Brockstedt, 1997).

In pan-fried pork, Sinha et al. (1998) reported on levels below the limit of detection of 0.2 ng/g for pork fried "just until done" and up to 1.3 ng MeIQx and 0.3 ng PhIP per g for pork fried "well done". Taking into account the specific mutagenicity of HAA in YG 1019, MeIQx (3800 revertants/ng) accounts for more than 99.9% of mutagenicity observed in fried pork, whereas the low levels of PhIP (5.0 revertants/ng) observed do not contribute to any significant extent. The mutagenicity extracted from the meat samples in this study varied between 110 and 3014 revertants per g fried meat corresponding to levels of 0.03–0.8 ng/g. Notably, the bioassay includes potentially unknown mutagenic compounds and is more sensitive as compared to conventional chemical analysis using HPLC with UV-detection.

It has been demonstrated that HAA of the aminoimidazo-azarene-type are formed from amino acids, reducing carbohydrates and creatine or creatinine as precursors

Table 3
Correlations between HAA, CP, pH and drip loss of the meat at various times post mortem

	HAA/CP	HAA/pH_1	HAA/pH_{15}	HAA/pH_{30}	HAA/pH_{45}	HAA/pH_{60}	HAA/pH_{1440}	HAA/Drip
Correlation	−0.051	0.126	−0.011	−0.011	−0.085	−0.061	−0.012	0.057
P-value	0.678	0.303	0.928	0.928	0.488	0.619	0.922	0.658

CP: creatine phosphate level (µmol/g fresh meat); HAA: heterocyclic aromatic amines, level of revertants per g fried meat in YG1019 with metabolic activation; pH_x: pH at x min post mortem; Drip: drip loss in %.

(Knize and Felton, 2005). This was demonstrated in model systems (Övervik et al., 1989; Jägerstad et al., 1991; Skog et al., 1992; Messner and Murkovic, 2004) and also in meat samples. Higher levels of mutagenicity were detected in fried meat with comparatively high levels of creatine, while lower levels were detected in meat with lower levels of creatine such as liver or kidney (Laser-Reutersward et al., 1987). Addition of creatine at 5% (w/w) to a meat emulsion model system resulted in a 40-fold increase of mutagenicity upon heating (Nes, 1986) and similar effects were reported in a model system with amino acids (threonine, glycine), glucose and creatine (Nes, 1987). Övervik et al. (1989) reported on an increase in mutagenicity of 2.4-fold or 4-fold upon addition of 2% or 5% creatine, respectively, to minced pork patties prior to broiling at 200 °C. The profile of HAA formed was not affected by addition of creatine. Felton et al. (1994) demonstrated that microwave pretreatment of meat reduced the level of precursors including creatine and diminished mutagenicity substantially.

However, others reported that there is no stoichiometric relation between creatine or creatinine concentrations in models systems and the formation of HAA (Jackson and Hargraves, 1995). Vikse and Joner (1993) reported on meats from different animals species, but observed no correlation between creatine content and mutagenicity upon frying, while reducing the creatine content enzymatically decreased also the mutagenic activity formed.

It has been proposed that creatinine rather than creatine is the actual precursor of HAA formation but it was demonstrated that creatinine is formed from creatine upon heating (Jackson and Hargraves, 1995).

Borgen et al. (2001) reported that higher levels of MeIQx are formed upon frying of a pork model system in the presence of water as compared to a dry heated system. However, the small variation of water content observed within the meat samples in this study had no effect on the formation of HAA.

Olsson et al. (2002, 2005) reported on the influence of different breeds and feeding regimes of pigs on the formation of HAA upon frying. They compared pork derived from pigs that were carriers and non-carriers of the RN-gene (Bertram et al., 2000). The RN mutation affects several of the precursors for the formation of HAAs, and due to accumulation of glycogen in glycolytic muscles, meat from carriers of the RN^- allele exhibits markedly higher reducing sugar levels, compared to the meat of non-carriers. Levels of mutagenic HAA were lower in fried meat from carriers of the RN^- allele but MeIQx levels were not significantly affected. This was attributed to the about fourfold higher glycogen level in meat from carriers of the RN^- allele. Olsson et al. (2002) demonstrated that the level of creatine in the meat is affected by the RN genotype of the pigs but no significant correlation was observed between creatine content and HAA formation (Olsson et al., 2002). Furthermore, no effects of feed (conventional or organic) on creatine levels in raw meat nor on HAA formation in fried meat were observed.

Here, we compared the meat from two different breeds, Duroc and Landrace pigs, both non-carriers of the RN^- allele (Rosenvold and Andersen, 2003). No significant difference was observed with regard to creatine content, measured as creatine phosphate or HAA content upon frying measured as mutagenicity of extracts (Table 1).

In an earlier report we demonstrated that meat from these two breeds differed with regard to water content, water holding capacity, colour determinants, pH at 24 h post mortem and sensory parameters such as juiciness (Young et al., 2005).

An increase in body weight gain was noted when pig feed was supplemented with CMH for five days prior to slaughter at dose levels of 12.5, 25 or 50 g per day. Generally, the effects of CMH feeding were more pronounced for meat from Duroc pigs as compared to meat from Landrace pigs: pH at 30 and 45 min post slaughter was higher while juiciness was reduced upon CMH supplementation. Here, we report that creatine phosphate levels in the meat increased with CMH supplementation, but this was only significant in meat from Duroc pigs (Fig. 2). Levels of mutagenicity were not affected, neither in meat from Duroc pigs nor in meat from Landrace pigs by CMH supplementation. Also, no effect on mutagen formation was associated with pH of the meat at 0.5–24 h post slaughter, drip loss or creatine phosphate content of the muscle. (Table 2).

Dietary creatine is being used as a legal ergogenic aid and has been shown to enhance athletic performance when taken at the recommended dosing regimen and as supplementation of an exercising program. Possible beneficial effects of creatine on the C-1 pool and homocysteine levels have been proposed, also experimental evidence has been presented on the protective effect of creatine in neurological disorders, such as ammonium induced neurotoxicity (Bachmann et al., 2004), MPTP-induced parkinsonism, Huntingdon's disease (Ryu et al., 2005) and a clinical trial on the pharmacotherapy of Parkinson's disease with creatine is being conducted (Meissner et al., 2004; Pena-Altamira et al., 2005).

Side effects that may result from high doses of creatine ingestion are of concern and have been subject to extensive scientific debate (Volek and Rawson, 2004).

Among the concerns are long term effects such as a hypothetical increase in carcinogenic risk that may result from an endogenous formation of HAA. Temperatures necessary to form these agents are reported to be 180 °C, and also prolonged cooking (100 °C) resulted in HAA formation but Klinae et al. (2005) report trace formation of HAA from model mixtures at 37 °C. A meal of meat has been shown to increase creatinine levels in plasma and urine (Jacobsen et al., 1980; Mayersohn et al., 1983). Recently, Holland and co-workers (2005) reported on the postprandial formation of a novel, weakly mutagenic HAA in human urine. The possible endogenous formation of HAA resulting from high levels of supplemental creatine consumed as ergogenic aid has not been investigated yet.

However, pig feed supplementation with CMH may increase weight gain and influence meat quality parameters. The effect on creatine levels in the meat is minimal and no influence on the formation of carcinogenic HAA detected as mutagenic activity was observed.

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Original Contribution

Salivary uric acid at the acidic pH of the stomach is the principal defense against nitrite-derived reactive species: Sparing effects of chlorogenic acid and serum albumin

Donatella Pietraforte [a,*], Marco Castelli [b], Alessio Metere [a], Giuseppe Scorza [a], Paola Samoggia [a], Antonio Menditto [b], Maurizio Minetti [a]

[a] *Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy*
[b] *Department of Food Safety and Veterinary Public Health, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy*

Received 5 April 2006; revised 26 July 2006; accepted 16 August 2006
Available online 22 August 2006

Abstract

A complex antioxidant system is present in human saliva, with uric acid being the most concentrated component. Ascorbic acid, present at low concentrations in saliva, is actively secreted into the gastric lumen. We report that ascorbic acid added to human saliva at pH 2 was consumed within a few minutes, regenerating HNO_2, whereas uric acid was consumed relatively slowly in a nitrite-dependent manner. The consumption of uric acid was (i) rapid under normoxic conditions and slower at low oxygen tensions, (ii) coupled to $^{\bullet}NO$ release, (iii) linked to the decrease in nitrite consumption and in nitrate formation, and (iv) unaffected by the nitrosation catalyst thiocyanate. Both chlorogenic acid and bovine serum albumin, representative of a phenol- and a protein-rich meal, respectively, were able to spare uric acid, although chlorogenic acid increased, whereas bovine serum albumin inhibited, $^{\bullet}NO$ release. We hypothesize that the major role of uric acid in saliva at pH 2 could be to preserve the stomach from the formation of toxic nitrogen species and that low levels of uric acid, together with ascorbic acid consumption, may contribute to the high occurrence of tumors at the gastroesophageal junction and cardia. The sparing effects of dietary compounds may therefore be an important not fully appreciated effect.
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Keywords: Uric acid; Reactive nitrogen species; Human saliva; Gastric cancer; Chlorogenic acid; Bovine serum albumin; Free radicals

The high incidence of tumors in the upper gastrointestinal tract has been linked to the formation of potentially mutagenic species derived from nitro(so)compounds. The formation of *N*-nitrosamines is well documented in the gastrointestinal tract [1,2] and depends on nitrite (NO_2^-) derived from inorganic nitrate (NO_3^-), which is found in large quantities in foods, particularly green leafy vegetables. The toxicity of NO_3^- is a matter of debate on account of concerns regarding the potentially carcinogenic effects of *N*-nitrosamines.

The metabolism of NO_3^- is complex and involves a symbiotic relationship with bacterial flora, through a process that seems to be tailored to the production of the bioactive molecule nitric oxide (nitrogen monoxide, $^{\bullet}NO$) [3–8]. NO_3^- ingested with foods is absorbed by the small intestine and a relevant fraction (about 25%) is concentrated in the salivary glands and resecreted into the mouth. Bacterial flora then reduce about 20–30% of NO_3^- to NO_2^-. NO_2^-, whose physiological role in biological fluids has only recently been appreciated [9,10], is present at relatively high levels in human saliva (HS), about 20–150 μM under fasting conditions and up to 1–2 mM after a NO_3^- loading equivalent to 100–200 g of green leafy vegetables. The acidification of NO_2^- in the stomach generates $^{\bullet}NO$ [3,4], a molecule with important biological functions, such as antimicrobial activity [5,11], gastric motility [12,13], the production of mucus, and the

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Abbreviations: HS, human saliva; GGWA, Gly-Gly-Trp-Ala tetrapeptide; GGYR, Gly-Gly-Tyr-Arg tetrapeptide; BSA, bovine serum albumin; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; NEM, *N*-ethylmaleimide; AH_2, ascorbic acid; SCN^-, thiocyanate ion.

[*] Corresponding author.
E-mail address: pietraf@iss.it (D. Pietraforte).

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doi:10.1016/j.freeradbiomed.2006.08.014

promotion of mucosal blood flow [14]. At the acidic pH of the stomach and in the presence of oxygen NO_2^- is present as nitrous acid (HNO_2, pK_a 3.2–3.4), a species with both radical and NO^+ donor characteristics (Scheme 1, Eq. (1)).

Saliva has a complex antioxidant system composed of peroxidases (salivary peroxidase and myeloperoxidase), superoxide dismutase activity and thiols, and low-molecular-weight compounds, including uric acid and lipid-soluble antioxidants such as α-tocopherol and β-carotene [15,16].

Uric acid is a physiologically important antioxidant not only in blood plasma [17] but also in saliva [15,16,18,19], where it contributes approximately 70% of the total antioxidant capacity. Conversely, ascorbic acid (AH_2) and serum albumin, which play a relevant antioxidant role in blood plasma, perform only a secondary role in HS on account of their relatively low concentrations [16,18]. The antioxidant properties of uric acid have been attributed to its ability to chelate transition metals, to react with biological oxidants such as the hydroxyl radical and hypochlorous acid, and to produce allantoin and oxonic acid as relatively stable products [20]. More recently, uric acid has been found to be an excellent scavenger of reactive nitrogen species [21–23] and an efficient inhibitor of peroxynitrite-mediated nitration of tyrosine residues both in vitro [23–25] and in vivo [26–29].

In addition to the above antioxidants, it has been suggested that polyphenol-rich foods and beverages may also perform a protective role against the mutagenic potential of nitrous acid by scavenging nitr(os)ating species [2,30,31]. Recently, we investigated the release of $^{\bullet}\mathrm{NO}$ in HS at the acidic pH of the stomach [32] and found that under aerobic conditions (i) some apple polyphenols promoted $^{\bullet}\mathrm{NO}$ release at the gastric level, (ii) AH_2 in saliva induced a SCN^--enhanced burst of $^{\bullet}\mathrm{NO}$ but the release was transient and led to HNO_2 regeneration, and (iii) apple polyphenols with a catechol group formed *o*-semiquinone radicals as intermediates and nitrated polyphenols as end products. The picture emerging from this study was that some foods can significantly modulate $^{\bullet}\mathrm{NO}$ homeostasis and prevent nitr(os)ation reactions in the gastrointestinal tract. In light of these results, we examined the fate of uric acid in HS at the acidic pH of the stomach. We found that uric acid was rapidly consumed in saliva as a function of pH and nitrite concentration. Our results suggest that the potential beneficial effects of uric acid were to promote the release of $^{\bullet}\mathrm{NO}$ in the stomach and to inhibit the formation of nitrogen dioxide radical ($^{\bullet}\mathrm{NO}_2$), a nitrating and oxidizing species. We also report the ability of chlorogenic acid and bovine serum albumin (BSA) to spare uric acid by scavenging reactive nitrogen species, suggesting that some foods may not only increase the bioavailability of uric acid but also significantly affect gastric $^{\bullet}\mathrm{NO}$ homeostasis.

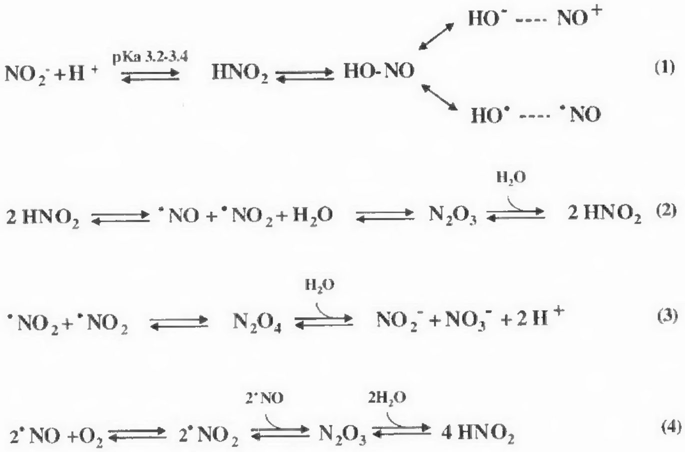
Materials and methods

Chemicals

The $^{\bullet}\mathrm{NO}$ donor $\mathrm{C_4H_{10}N_3O_2Na}$ (DEA-NO) was obtained from Alexis Corp. (Laufelfingen, Switzerland). Methanol (99.8%; highest grade purity) was purchased from J.T. Baker (Deventer, Holland). Uric acid was purchased from the National Institute of Standards and Technology (Gaithersburg, MD, USA). Gly-Gly-Trp-Ala (GGWA) and Gly-Gly-Tyr-Arg (GGYR) were from Serva Feinbiochemia GmbH (Heidelberg, Germany) and Pronase was from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents were from Sigma–Aldrich (St. Louis, MO, USA). Distilled water used in all experiments was previously treated with Chelex 100 (Bio-Rad, Richmond, CA, USA) to remove traces of transition metals.

Collection and preparation of HS

To obtain HS with different NO_2^- contents, HS was collected from healthy volunteers, after overnight fasting, before or 1 h after the ingestion of a meal composed of 100 g of a salad of mixed green vegetables, such as lettuce, rocket, and spinach. Before HS was collected, the mouth was washed three times with water. The collection of HS was initiated 1 h after the ingestion of salad and continued for 1 h. HS was maintained at 0°C during collection and was centrifuged at



Scheme 1. Chemistry of acidic nitrite in aerobic solutions.

12,000 *g* for 10 min, filtered (0.2 μm), and immediately stored at −80°C until use. The range of uric acid concentrations in both fasting and postmeal HS samples was 245.5–354.6 μM ($n=15$), in good agreement with data reported elsewhere [18]. Furthermore, the ranges of NO_2^- and NO_3^- concentrations in fasting HS were 50–253 and 270–340 μM, respectively, whereas after a salad meal the concentrations of NO_2^- and NO_3^- rose to 1.01–1.58 and 1.96–2.12 mM, respectively. Unless noted otherwise, all experiments were performed under aerobic conditions and at 37°C. To acidify the samples, HCl was added to obtain the desired pH. The acidification of hypoxic samples was obtained by adding previously degassed HCl under a nitrogen flux. When necessary, HS was exhaustively dialyzed (cutoff 3000) to remove low-molecular-weight compounds. HS enriched with BSA was prepared by suspending the BSA powder at the desired concentration directly in HS. Sulfhydryl alkylation of BSA (1 mM) was performed by preincubation with 10 mM *N*-ethylmaleimide (NEM) in isotonic phosphate buffer, pH 7.4, for 1 h at 37°C, followed by extensive dialysis against the same buffer. Alkylated BSA was then lyophilized and dissolved in HS. The efficiency of BSA alkylation was 93%, as measured with 5,5′-dithiobis 2-nitrobenzoic acid ($\varepsilon_{412}=13{,}600\ M^{-1}\ cm^{-1}$).

HPLC determination of uric acid content in HS

Aliquots of HS samples incubated at 37°C were diluted first 1:1 with 1 M phosphate buffer (pH 7.4) and then with methanol (1:5) and centrifuged for 1 min (15,000 rpm). The samples were then filtered through a 0.22-μm syringe filter and a volume of 20 μl was injected into the HPLC. Uric acid was determined by reverse-phase HPLC (Supelcosil LC-18 column and guard column Supelcosil LC-18 Supelguard cartridge; Sigma–Aldrich, Steinheim, Germany) with spectrophotometric detection at 265 nm as reported in [33]. The mobile phase consisted of dodecyltrimethylammonium bromide 7 mM and ammonium formate 1 mM in methanol:water (60:40, v/v), pH 5.9.

HPLC determination of nitrite and nitrate

NO_2^- and NO_3^- concentrations were determined by HPLC (IonPac Dionex AS9-HC column; Dionex, Austin, TX, USA). For the experiments performed in the presence of BSA, measurements were performed in the ultrafiltrate (Microcon concentrators, cutoff 3000; Amicon, Beverly, MA, USA).

Electrochemical detection of $^{\bullet}NO$

$^{\bullet}NO$ release was measured at room temperature with a Clark-type electrode (Iso-NOP, Apollo 4000; World Precision Instruments, Sarasota, FL, USA) under constant stirring and in open vessels or, when necessary, under a continuous N_2 flux. After stabilization of the baseline, the reaction was started by the addition of HCl (final pH value 2.0±0.2). DEA-NO ($\varepsilon_{250\ nm}=6.5\ mM^{-1}\ cm^{-1}$) was used to calibrate the electrode (1 pA=1.47±0.5 nM $^{\bullet}NO$, $n=5$).

Spectrophotometric measurements of uric acid consumption under hypoxic conditions

To reduce the concentration of dissolved oxygen, uric acid solution or HS was gently bubbled with N_2 for 60 min at room temperature. Degassed samples were then acidified under N_2 bubbling with previously degassed HCl and the absorption of uric acid at 285 nm was monitored in closed vials at 37°C.

Nitrotyrosine

After incubation (30 min, 37°C) at acidic pH, samples were neutralized by adding 1:1 (v/v) 1.0 M phosphate buffer, pH 7.4, and processed for the immunodetection of 3-nitrotyrosine as reported by Ferroni et al. [34].

EPR spectroscopy

Spectra were measured on a Bruker ECS 106 spectrometer equipped with a variable-temperature unit (ER4111VT). Samples were drawn up into a gas-permeable Teflon tube with 0.81-mm internal diameter and 0.05-mm wall thickness (Zeuss Industrial Products, Raritan, NJ, USA). The Teflon tube was folded four times, inserted into an open (air) quartz tube, and fixed to the ESR cavity (4108 TMH). The temperature was 37°C. All ESR spectra were corrected for baseline drift by a linear function and double integrated and simulated using the software supplied by Bruker (ESP 1600 data system). Spectrometer conditions common to all spectra were modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; microwave power, 20 mW.

Results

Fate of ascorbic and uric acids in HS at pH 2.0

AH_2, the most important antioxidant defense in blood plasma, is present at low levels in HS (median value 0.5 μM [35]) but is actively secreted into the gastric lumen, reaching a concentration of 20–300 μM [36–38]. AH_2 is known to promote the release of $^{\bullet}NO$ when HS meets the acidic pH of the stomach [39]. However, because of its rapid reaction with HNO_2, AH_2 is immediately consumed in the upper part of the stomach [1,36,39,40] with the result that its role is restricted to the beginning of the reaction. The $^{\bullet}NO$ is then adsorbed by gastric mucosa or reacts with oxygen, regenerating nitrous acid and favoring both the depletion of AH_2 and the consumption of oxygen [1,36,39,40]. In agreement with these observations, our HPLC measurements indicated that AH_2 was rapidly depleted in HS (70±10 μM/min; $n=3$). These results, further confirmed by EPR studies of AH_2 oxidation through the formation of the ascorbyl radical (results not shown), suggest that even under fasting conditions AH_2 is depleted in the stomach within a few minutes, although this reaction is physiologically relevant for a continuous supply of $^{\bullet}NO$ [41–43].

Due to the rapid consumption of AH_2 in HS at the acidic pH of the stomach, which limits its antioxidant role, it was

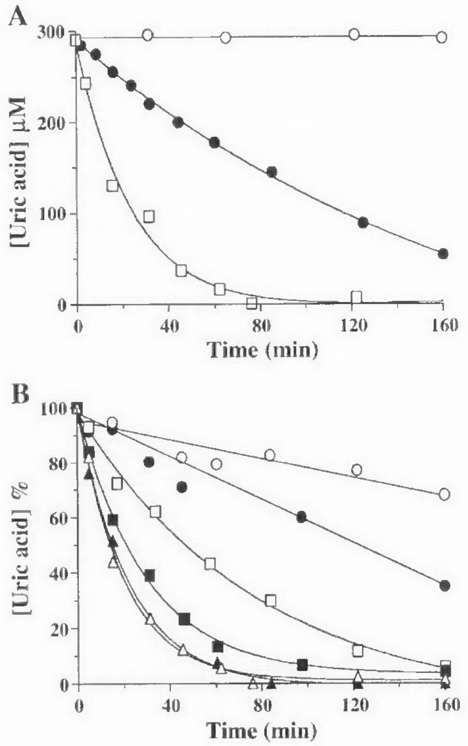


Fig. 1. Time course of uric acid consumption in HS at 37°C. (A) Effects of pH. Uric acid content measured in HS incubated at pH 7.4 (open circles) or after acidification at pH 3.8 (closed circles) or pH 1.9 (open squares). Only one representative experiment of three is shown. (B) Effects of NO_2^- concentration. HS containing 0.12 (open circles), 0.24 (closed circles), 0.47 (open squares), 0.89 (closed squares), 1.1 (open triangles), and 1.3 mM (closed triangles) NO_2^- was incubated at pH 1.95. Data are expressed as % of uric acid present in HS before acidification. Only one representative experiment of three is shown.

interesting to study the fate of uric acid, the most concentrated antioxidant of HS. The time course of uric acid consumption was first investigated by incubating HS (1.10 mM as NO_2^-) at 37°C and at various pH (Fig. 1A). At pH 7.4, the uric acid content was not modified, at least within 2 h. However, if the reaction was performed at pH 3.8, the uric acid content decreased rapidly and after 160 min its concentration was reduced by about 86%. Interestingly, the acidification of HS to pH 1.9, which is close to that found in the stomach, induced a further increase in the rate of uric acid consumption, so that it was exhausted within 80 min (Fig. 1A).

This pH dependence indicates that uric acid consumption does not occur in the oral cavity but in the acidic conditions of the stomach. Bearing in mind that the NO_2^- content of HS changes as a function of food intake, the consumption of uric acid at the acidic pH of the stomach is expected to be dependent on NO_2^- concentration. Fig. 1B shows the time course of uric acid consumption in HS as a function of the HNO_2 concentration. To avoid the dilution of uric acid and, at the same time, the modification of HS composition, samples of HS containing different NO_2^- concentrations were obtained by mixing (v/v) fasting and postmeal HS. From the time course reported in Fig. 1B, we calculated that in HS with a concentration of 0.12 mM NO_2^- the time necessary to halve the uric acid content (*t*/2) was about 270 min. The *t*/2 was decreased when the NO_2^- concentration was increased: *t*/2 = 125, 42, 22, 12, and 10 min for 0.24, 0.47, 0.89, 1.10, and 1.3 mM NO_2^-, respectively.

Effects of uric acid on $^\bullet NO$, NO_2^-, and NO_3^-

The $^\bullet NO$ released by acidified postmeal HS was too high to be measured by the $^\bullet NO$ electrode and, moreover, the measure of NO_3^- formation and NO_2^- consumption was not very accurate on account of the high basal levels of these ions (1.96–2.12 and 1.01–1.58 mM, respectively); we therefore studied the effects of uric acid on these species in a NO_2^- solution at pH 2.0. The acidification of 0.1 mM NO_2^- solution under aerobic conditions induced a steady-state level of 0.367 μM $^\bullet NO$ (Fig. 2A, trace a). The generation of $^\bullet NO$ lasted for at least 45 min without decline, suggesting that the $^\bullet NO$ level was achieved through a continuous recycling of $^\bullet NO$ to HNO_2. Interestingly, in the presence of uric acid, $^\bullet NO$ release from acidic NO_2^- solution increased to 1.05 and 1.75 μM $^\bullet NO$ for 100 and 250 μM uric acid (Fig. 2A, traces b and c, respectively). In oxygenated

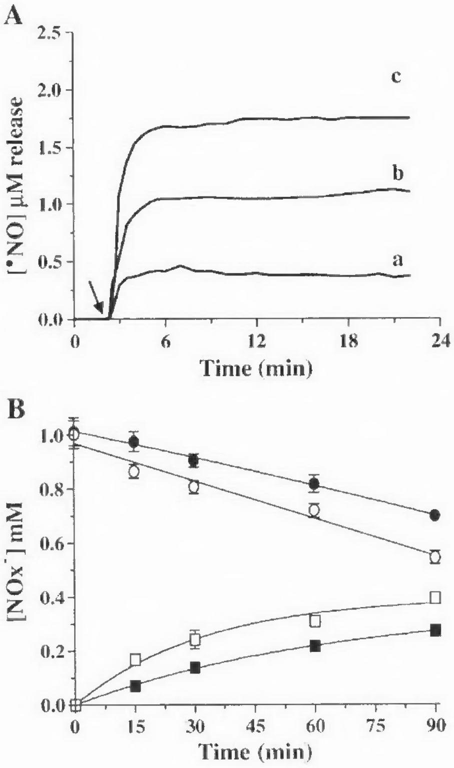


Fig. 2. Effects of uric acid on the nitrogen oxides in acidic NO_2^- solution. (A) $^\bullet NO$-promoting activity of uric acid. The arrow indicates acidification with HCl at pH 1.9. NO_2^- (100 μM) was incubated in the absence (trace a) or in the presence of 100 (trace b) or 250 μM (trace c) uric acid. Only one representative experiment of three is shown. (B) Kinetics of NO_2^- concentration (circles) and NO_3^- formation (squares) in a NO_2^- (1.0 mM) solution incubated at pH 2.0 and 37°C in the absence (open symbols) and in the presence (closed symbols) of 250 μM uric acid. Because neutralization converts $^\bullet NO$ and all HNO_2-derived species to NO_2^- the indicated concentration of NO_2^- is actually the sum of all these species. Points represent mean values ± SD ($n = 3$).

samples, the species in equilibrium with HNO_2 are $^{\bullet}NO$, N_2O_3, $^{\bullet}NO_2$, and N_2O_4 as shown by Eqs. (2)–(4) of Scheme 1. N_2O_3 is considered responsible for the nitrosating activity of HNO_2, whereas $^{\bullet}NO_2$, which is converted to N_2O_3 by a fast radical–radical reaction with $^{\bullet}NO$, is responsible for the nitrating/oxidizing capacity of HNO_2. HS contains, in addition to urate and NO_2^-, millimolar concentrations of SCN^-, which, through the formation of a transient species (ONSCN), is a strong catalyst of the nitrosation reaction [44]. As a consequence of the presence of SCN^-, HS seems to be forced toward nitrosating reactions. For example SCN^- has been reported to enhance severalfold the AH_2 oxidation rate in acidic solutions of NO_2^- [32,41]. Therefore, to understand the antioxidant mechanism of uric acid, we investigated the effects of SCN^-. By using both HPLC and spectrophotometric analysis, we studied in depth the role of SCN^- on urate consumption in a NO_2^- solution at pH 2.0. Both techniques showed that SCN^- did not appreciably affect the rate of urate depletion (results not shown). These results suggest that urate consumption in acidic HS is not due to a SCN^--catalyzed nitrosation reaction and suggest the hypothesis that urate is consumed through a reaction with a HNO_2-derived species other than N_2O_3.

At neutral pH, NO_2^- is stable and its concentration does not change over time, whereas at acidic pH and in open-air-equilibrated solutions it slowly declines on account of the loss of $^{\bullet}NO$ as gas and the formation of nitrate. As shown in Fig. 2B, the concentration of 1.0 mM NO_2^- solution at pH 2.0 halved in about 90 min. It should be emphasized that the measured concentration of NO_2^- is actually the sum of NO_2^- and other species (such as HNO_2, $^{\bullet}NO$, $^{\bullet}NO_2$, N_2O_3, N_2O_4) because the latter species are converted to NO_2^- by neutralization before measurement (we refer to this as the "NO_2^- concentration," but it is actually the sum of all these species). In the presence of 250 μM uric acid, the rate of NO_2^- consumption was appreciably slowed from 5.05 to 3.3 μM/min (Fig. 2B). Measurement of the time course of NO_3^- formation performed in the same samples indicated that 0.341 mM NO_3^- was produced after 90 min incubation at pH 2.0, whereas in the presence of uric acid the formation of NO_3^- was inhibited by about 26% (Fig. 2B). It should be emphasized that: (i) the formation of NO_3^- is indicative of the production of $^{\bullet}NO_2$ because in aerobic solutions $^{\bullet}NO$ autoxidizes to NO_2^- [45] and (ii) the sum of $NO_2^-+NO_3^-$ was similar irrespective of the presence of uric acid (0.978 ± 0.02 and 0.913 ± 0.03 mM, respectively). These data indicate that uric acid scavenges $^{\bullet}NO_2$, as suggested by the decrease in NO_3^-, and, as a consequence, shifts the reaction toward the formation of $^{\bullet}NO$, which in oxygenated solutions autoxidizes to HNO_2 (Scheme 1, Eq. (4)).

Effects of oxygen

In acidic NO_2^- oxygen causes a recycling of HNO_2, but the fraction of $^{\bullet}NO$ released as gas leads to a slow accumulation of NO_3^- through N_2O_4 dismutation (Eq. (3)). Consequently, the hypoxic conditions should be less favorable for the formation of nitr(os)ating species. The consumption of uric acid would therefore be expected to be slower if its oxidation depends on $^{\bullet}NO_2$. We performed kinetics studies of uric acid consumption under hypoxic conditions in both NO_2^- solution and HS at pH 2.0 (Figs. 3A and B, respectively). The reduction in the oxygen concentration effectively led to a slowdown in the uric acid oxidation rate (decrease of 56.7 and 40.3% in NO_2^- solution and HS, respectively). As shown in Fig. 3B, the acidification of HS led to a transient increase in absorbance at 285 nm under both normoxic and hypoxic conditions, which was not observed in the NO_2^- solution. This phenomenon is due to a pH-dependent hyperchromic shift of a still unidentified low-molecular-weight component present in HS (cutoff 3000; our unpublished results).

The decrease in uric acid consumption under hypoxic conditions clearly demonstrates that the oxidizing species is formed during $^{\bullet}NO$ autoxidation, supporting our hypothesis that the antioxidant mechanism is the scavenging of $^{\bullet}NO_2$. Moreover, the gastrointestinal tract is largely hypoxic [1,39] and it is therefore to be expected that uric acid consumption is limited to the first aerobic step after the swallowing of saliva.

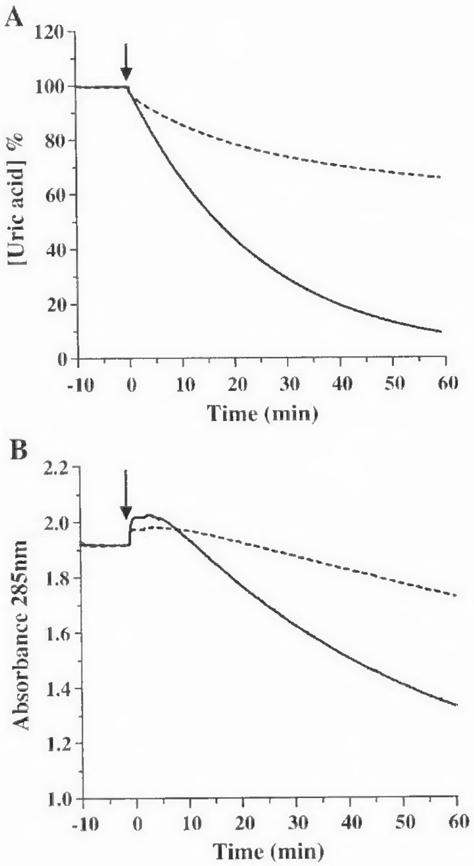


Fig. 3. Effects of oxygen depletion on uric acid in a NO_2^- solution or in HS at pH 2.0. (A) Uric acid consumption in a NO_2^- solution. Air-equilibrated (solid line) and N_2-bubbled (dotted line) solutions are represented. NO_2^- (1.0 mM) and uric acid (150 μM) were incubated at pH 2.0 and 37°C. The arrow indicates acidification with HCl. Only one representative experiment of three is shown. (B) Uric acid consumption in HS. Air-equilibrated (solid line) or N_2-bubbled (dotted line) solutions are represented. HS was diluted 1:3 in water (122 μM as uric acid and 0.450 mM as NO_2^-) and incubated at pH 2.0 and 37°C. The arrow indicates acidification with HCl. Only one representative experiment repeated in triplicate is shown.

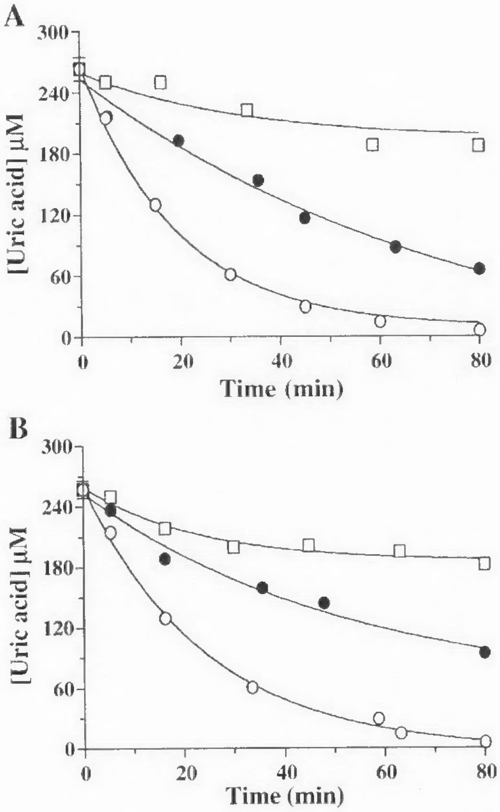


Fig. 4. Effects of chlorogenic acid and BSA on HS uric acid consumption. (A) Effects of chlorogenic acid. HS (1.1 mM as NO_2^-) was incubated at 37°C at pH 2.0 in the absence (open circles) or in the presence of 0.5 (closed circles) or 1 mM (open squares) chlorogenic acid. Only one representative experiment repeated in triplicate is shown. (B) Effects of BSA. HS (1.1 mM as NO_2^-) was incubated at 37°C at pH 2.0 in the absence (open circles) or in the presence of 0.5 (closed circles) or 1 mM (open squares) BSA. Only one representative experiment repeated in triplicate is shown.

Comparison of uric acid-sparing effects of chlorogenic acid and bovine serum albumin

In a previous paper we reported the ability of chlorogenic acid to promote the release of $^{\bullet}$NO from acidified HS and to scavenge $^{\bullet}\text{NO}_2$ [32]. This phenolic antioxidant, present at high concentrations in some fruits and vegetables, is able to scavenge $^{\bullet}\text{NO}_2$ through the formation of nitrated chlorogenic acid with the *o*-semiquinone radical being an intermediate [32]. Of the high-molecular-weight antioxidants, serum albumin is one of the most important in extracellular fluids, although its antioxidant role is due largely to its high concentration, for which reason it is considered a "suicidal antioxidant." Its concentration in HS is, however, significantly lower than that in serum (13 and 600 µM, respectively [18]) and it is thus considered of secondary relevance as an antioxidant in HS. However, BSA is widely present in foods and can be considered representative of a protein-rich meal. Importantly, nitr(os)ation reactions of BSA have been previously described in acidic NO_2^- solutions [46–48]. Taken together, these considerations suggest that chlorogenic acid and BSA may have a role in both $^{\bullet}$NO homeostasis and uric acid consumption, although the experimental proof is lacking.

Fig. 4A shows that chlorogenic acid strongly reduced uric acid depletion in HS (1.10 mM as NO_2^-) at pH 2.0. In particular, after 80 min at 37°C, the residual uric acid content of acidified HS was 7.5, 65.8, and 186.2 µM in the presence of 0, 0.5, and 1 mM chlorogenic acid, respectively. Uric acid was equally preserved in a dose-dependent manner when HS was acidified in the presence of BSA (Fig. 4B). After 80 min at 37°C, the residual uric acid was increased to 94.6 and 182.3 µM at 0.5 and 1 mM BSA, respectively. We conclude that chlorogenic acid and BSA have comparable sparing effects on uric acid.

Comparison of the effects of chlorogenic acid and bovine serum albumin on $^{\bullet}$NO, NO_2^-, and NO_3^-

The strong $^{\bullet}$NO-promoting activity of chlorogenic acid in acidified HS (0.050 mM as NO_2^-) is shown in Fig. 5A. When this polyphenol was added to HS, even in very low concentrations, the $^{\bullet}$NO steady-state level increased considerably. Unlike chlorogenic acid, BSA did not promote the $^{\bullet}$NO release from acidified HS (0.17 mM as NO_2^-), but inhibited it at high concentrations (Fig. 5B). The decrease in $^{\bullet}$NO release by 0.2, 0.5, and 1 mM BSA was 16, 45, and 83% respectively.

As we previously reported [32], chlorogenic acid accelerated NO_2^- consumption and inhibited NO_3^- formation. As

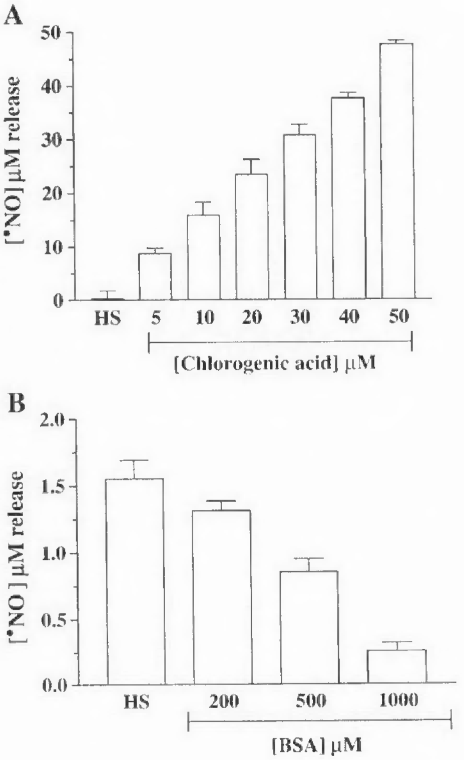


Fig. 5. Comparison of the effects of chlorogenic acid and BSA on $^{\bullet}$NO release of HS. (A) Effects of chlorogenic acid. HS (0.05 mM as NO_2^-) was incubated in the absence or in the presence of chlorogenic acid at 37°C, pH 1.9. (B) Effects of BSA. HS (0.17 mM as NO_2^-) was incubated in the absence or in the presence of BSA at 37°C, pH 1.9. $^{\bullet}$NO concentration was measured 20 min after acidification. Points represent mean values ± SD ($n=3$).

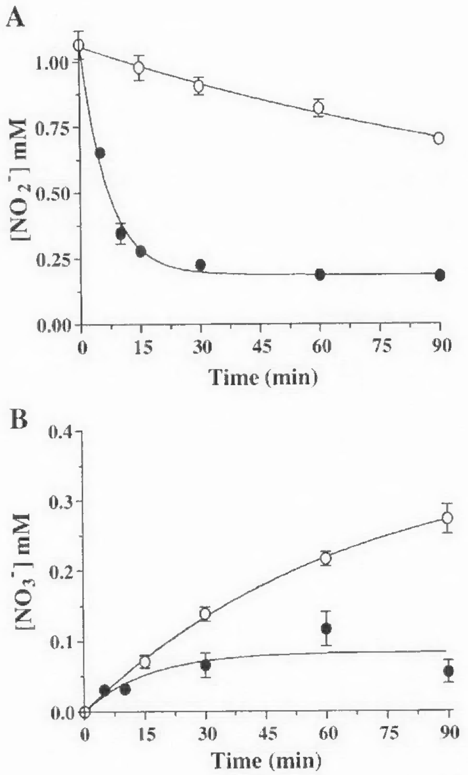


Fig. 6. Effects of BSA on NO_2^- concentration and NO_3^- formation. (A) Kinetics of NO_2^- concentration in a NO_2^- (1 mM) and uric acid (250 μM) solution incubated at pH 1.9 at 37°C, in the absence (open circles) or in the presence of 1 mM BSA (closed circles). (B) Kinetics of NO_3^- formation in a NO_2^- (1 mM) and uric acid (250 μM) solution incubated at pH 1.9 at 37°C, in the absence (open circles) or in the presence of 1 mM BSA (closed circles). Because neutralization converts ${}^{\bullet}NO$ and all HNO_2 species to NO_2^-, the indicated concentration of NO_2^- is actually the sum of all these species. Points represent mean values ± SD (n=3).

shown in Figs. 6A and 6B, the addition of BSA to a NO_2^- solution containing 250 μM uric acid at pH 2.0 also induced the acceleration of NO_2^- consumption and the inhibition of NO_3^- formation. The consumption of NO_2^- in the presence of uric acid was dramatically accelerated in the presence of BSA, following a rapid kinetics in the first 15 min (80% NO_2^- consumed), but complete consumption of NO_2^- was not reached even after 90 min. A possible explanation for this phenomenon may be that under these conditions BSA is nitrosated [46–48], but after alkalinization for the HPLC analysis some ${}^{\bullet}NO$ is released and autoxidizes to NO_2^-. As shown in Fig. 6B, the formation of NO_3^- in these samples was also clearly inhibited when BSA was included in the reaction (80% inhibition after 90 min).

BSA forms a protein-centered radical and nitrotyrosines in HS at pH 2.0

The antioxidant activity of BSA is linked to the presence of some amino acid residues, such as Cys, Met, Tyr, and Trp, able to react rapidly with reactive oxygen and nitrogen species. Because in the reaction with reactive nitrogen species protein-centered radicals can be formed as intermediates, we performed EPR spin trapping experiments to characterize the radicals involved. Fig. 7, spectra A–E, shows the results obtained with the spin trap 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS). Spectrum A shows that acidification of HS (1.10 mM as NO_2^-) in the presence of DBNBS induced the formation of a partially immobilized adduct of weak intensity due to the trapping of radicals

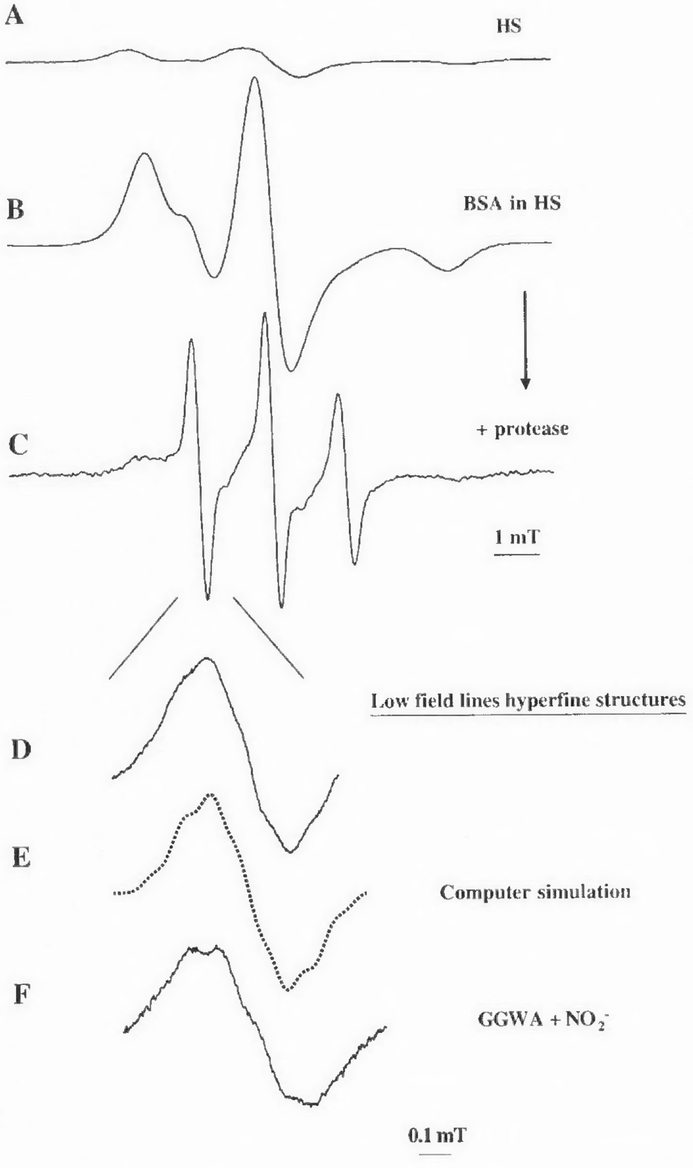


Fig. 7. Spin trapping with DBNBS of radicals formed in HS at pH 2.0 in the presence of BSA. Spectrum A: HS (1.1 mM as NO_2^-) in the presence of DBNBS (5 mM). Spectrum B: sample as in A, but in the presence of BSA (1 mM). Spectrum C: sample as in B, but 5 min after neutralization with phosphate buffer (0.1 M), pH 7.0, and addition of Pronase (5 mg/ml). Spectrum D: superhyperfine structure of the low field line of sample C. Spectrum E: computer simulation using a_N=0.053, a_H=0.094, a_H=0.095, a_H=0.059, $2a_H$ *meta*=0.12 mT. Spectrum F: GGWA tetrapeptide (9 mM) in the presence of DBNBS (5 mM), pH 2.0. Spectra were acquired at 37°C. Spectra were acquired 1 min after acidification. Instrument settings were as follows: modulation amplitude, 2 (spectra A, B), 1 (spectrum C), and 0.1 G (spectra D, F); time constant, 165 (spectra A–C) and 328 ms (spectra D, F); sweep time, 42 (spectra A–C) and 84 s (spectra D, F); number of scans, 5 (spectra A, B), 1 (spectrum C), and 20 (spectra D, F).

centered on proteins present in HS. When BSA was included in the reaction, an intense and strongly immobilized adduct was detected (spectrum B). Alkylation of BSA Cys residues by NEM did not decrease but increased (about 60%) the DBNBS adduct intensity (spectrum not shown), excluding the trapping of cysteinyl radicals (in this case the alkylation is inhibitory). The proteolytic digestion of the DBNBS–BSA adduct generated a nearly isotropic signal (spectrum C) composed of a three-line spectrum ($a_N=1.336\pm0.014$ mT) characteristic of a tertiary carbon-centered radical. Both Tyr and Trp have been described as forming DBNBS adducts with a similar hyperfine structure [49,50] and we therefore studied the superhyperfine structure of the low field line that, as previously demonstrated by Gunther et al. [49], can unambiguously identify the radicals. As shown in spectrum D of Fig. 7, the superhyperfine structure of the Pronase-digested adduct of DBNBS–BSA is similar to that of a Trp radical [49]. Accordingly, the superhyperfine structure was computer-simulated assuming a Trp radical centered on the C_3 position of the indolic ring (Fig. 7, dotted spectrum). To confirm further the trapping of a Trp radical, we compared the superhyperfine structures of the low field line of the DBNBS adducts obtained by treatment with NO_2^- at pH 2.3 of the tetrapeptides GGWA and GGYR. The DBNBS–GGWA adduct showed the expected three-line isotropic signal assigned to a tertiary carbon-centered radical (hyperfine structure, $a_N=1.336\pm0.014$ mT; spectrum not shown) with a superhyperfine structure similar to that observed in protease-treated DBNBS–BSA adduct (Fig. 7, spectrum D) [49]. However, the incubation of GGYR with NO_2^- at pH 2.3 in the presence of DBNBS generated a weak three-line isotropic signal due not to the trapping of a Tyr radical but to an oxidation product of DBNBS as demonstrated by the incubation of DBNBS with NO_2^- at pH 2.3 without the tetrapeptide ($a_N=0.967\pm0.017$ mT) (spectra not shown).

Although it has been claimed that Tyr-centered radicals generate DBNBS adducts with a_N values similar to those generated by Trp-centered radicals [50], in our samples we did not find evidence of Tyr radical formation. This result seems to be in contradiction to previous reports showing that BSA incubated with NO_2^- at acidic pH induced the formation of nitrotyrosines [47,51], which is considered the product of the fast radical–radical reaction between Tyr radicals and $^{\bullet}NO_2$ ($k=3.0\times10^9$ M^{-1} s^{-1}). To solve this inconsistency, the formation of nitrotyrosine in BSA was investigated by Western blot analysis using anti-3-nitrotyrosine antibodies (Fig. 8). A nitrotyrosine band corresponding to BSA was revealed when the protein was incubated in a NO_2^- solution at pH 2.0 (Fig. 8A, lane 1). Interestingly, the BSA nitration was decreased by about 40% when 250 μM uric acid was included in the reaction (lane 2), confirming the ability of this antioxidant to scavenge the $^{\bullet}NO_2$. When BSA was incubated in acidified HS (Fig. 8A, lane 3) the inhibition of BSA nitration was about 70%. The importance of the HS low-molecular-weight antioxidant system, including uric acid, was highlighted by the finding that nitration of BSA was increased by 250% when the incubation was performed in dialyzed HS reconstituted with NO_2^- (Fig. 8B).

Discussion

Mechanism of uric acid reaction with HNO_2-derived species

The ability of uric acid to act as an antioxidant [17,52] was known long before it was recognized in vitro as an efficient scavenger of reactive nitrogen species such as $^{\bullet}NO$, $ONOO^-$, and $^{\bullet}NO_2$ [21,22,53–55]. Uric acid has been shown to prevent the pathological consequences of the formation of $^{\bullet}NO$-derived species ex vivo in human plasma [23,56] and in vivo in animal models [26,29], although this scavenging activity leads to its depletion in tissues [56,57].

In a recent paper, Takahama et al. [58] reported that $^{\bullet}NO$-derived species are also continuously formed in the oral cavity, on account of the bacteria-dependent NO_2^- reduction at neutral pH [59]. In this work, we report that at the acidic pH of the stomach and under oxygenated conditions salivary uric acid is a critical scavenger of reactive nitrogen species. The finding that BSA nitration is inhibited to a greater extent in HS than in NO_2^- solution and that uric acid has an inhibitory effect (Fig. 8) provides further evidence of the protective role of this antioxidant. The hypothesis that uric acid is one of the most important antioxidants of HS at the acidic pH of the stomach is strengthened by (i) its high concentrations, (ii) its fast reaction with HNO_2-derived species, and (iii) its time- and pH-dependent consumption (Fig. 1).

The NO_2^- chemistry in acidic conditions proceeds through the formation of HNO_2 (pK_a 3.2–3.4), a compound with the characteristics both of NO^+/OH^- and of the radical couple $^{\bullet}OH/^{\bullet}NO$ (Eq. (1)). In oxygenated solutions, the autoxidation of $^{\bullet}NO$ generates other nitrogen oxides with $^{\bullet}NO_2$ and N_2O_3 as intermediates (Eq. (4)). The diffusion-controlled reaction between $^{\bullet}NO$ and $^{\bullet}NO_2$ (1.1×10^9 M^{-1} s^{-1} [60]) is significantly faster than the reaction of $^{\bullet}NO$ with O_2 ($k=2.4\times10^6$ M^{-2} s^{-1} [61]) and leads to the formation of the nitrosating agent N_2O_3 (Eq. (4)). This explains the predominant nitrosating properties of HNO_2 because $^{\bullet}NO_2$ is a nitrating species, whereas N_2O_3 possesses nitrosating but not nitrating activity. Although the $^{\bullet}NO/^{\bullet}NO_2$ reaction leads to rapid regeneration of HNO_2, a small fraction of $^{\bullet}NO$ escapes this reaction, allowing $^{\bullet}NO_2$ to dimerize to NO_2^- and NO_3^- (Scheme 1, Eq. (3)). It is important to emphasize that $^{\bullet}NO_2$ is the only source of NO_3^-, which is also a proof of $^{\bullet}NO_2$ formation.

At neutral pH, 99% of uric acid exists as a single species (the monoanion) with a p*K*a of 5.4. At the acidic pH of the stomach, the urate monoanion becomes protonated (UH_3) and this is the species expected to react with NO_2^--derived compounds. Interestingly, four elements point to $^{\bullet}NO_2$ as the likely NO_2^--derived compound reacting with uric acid. First, the inhibition of uric acid consumption obtained under reduced oxygen tensions suggests that the oxidizing species is formed during the $^{\bullet}NO$ autoxidation (Fig. 3). Second, as previously proposed [52,54], in the reaction with uric acid $^{\bullet}NO_2$ can act as a one-

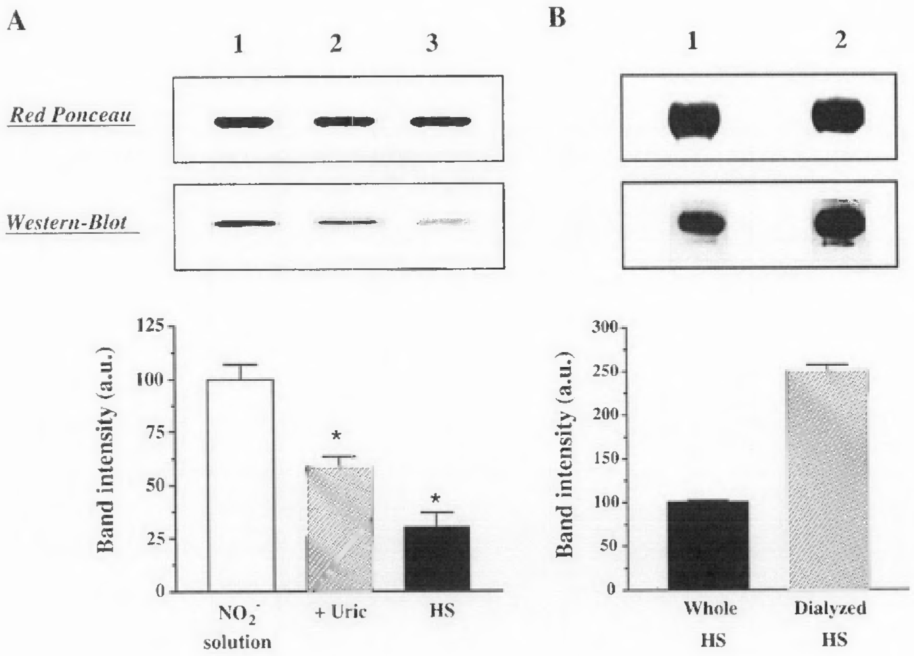


Fig. 8. Formation of BSA–nitrotyrosine in HS. (A) Comparison of BSA nitration occurring in HNO_2 solution and in HS. Top: Red Ponceau staining and Western blot analysis with anti-3-nitrotyrosine antibody of BSA (1 mM) incubated 30 min, 37°C, pH 2.0, with (lane 1) NO_2^- (1 mM), (lane 2) NO_2^- (1 mM) and uric acid (250 μM), and (lane 3) HS (1.1 mM as NO_2^-). Each lane was loaded with 5 μg of protein. Data are representative of three different experiments. (B) Effects of HS low-molecular-weight compounds on BSA nitration. Top: Red Ponceau staining and Western blot analysis with anti-3-nitrotyrosine antibodies of BSA (1 mM) incubated 30 min, 37°C, pH 2.0, with (lane 1) whole HS (1.1 mM as NO_2^-) and (lane 2) dialyzed HS reconstituted with NO_2^- (1.1 mM). Each lane was loaded with 20 μg of protein. Bottom: Densitometric analyses of the Western blot bands. Data are representative of three different experiments. $^*p \leq 0.01$ vs control.

electron oxidant with formation of the urate radical and HNO_2 (Eq. (5)). In agreement with this reaction scheme we found that uric acid slows down the rate of NO_2^- consumption in aerobic solutions (Fig. 2B):

$$UH_3 + {}^{\bullet}NO_2 \rightarrow UH_2^{\bullet -} + HNO_2^- . \qquad (5)$$

Third, the uric acid/$^{\bullet}NO_2$ reaction is expected to inhibit $^{\bullet}NO_2$ dismutation to NO_3^- and to increase the release of $^{\bullet}NO$. Both these hypotheses are supported by experimental results (Fig. 2). Fourth, the fact that uric acid consumption in acidified HS was not modified in the presence of SCN^- is also consistent with this hypothesis. The lack of effects of SCN^- indicates that uric acid does not compete with SCN^- for HNO_2 and does not react with the nitrosating intermediate NOSCN.

The rate constant of $^{\bullet}NO_2$ with uric acid is lower ($k = 1.8$–$2 \times 10^7\ M^{-1}\ s^{-1}$ [52,54]) than that with $^{\bullet}NO$ ($k = 1 \times 10^9\ M^{-1}\ s^{-1}$ [60]). For this reason the scavenging reaction of $^{\bullet}NO_2$ by uric acid is not complete, thus explaining the relatively modest inhibition of NO_3^- formation. It should be borne in mind, however, that these rate constants were calculated at pH 7.4 and may be different at acidic pH. Further, the concentration of uric acid in HS (240–305 μM) is about 600 times higher than the steady-state concentration of $^{\bullet}NO$ as measured electrochemically (about 0.4 μM; Fig. 2A), thus rendering the uric acid/$^{\bullet}NO_2$ reaction plausible.

It could be expected that the urate radical formed in Eq. (5) might react rapidly with $^{\bullet}NO$ and/or $^{\bullet}NO_2$ to form nitroso- or nitro-derivatives (R–ONO or R–NO_2). Interestingly, Skinner et al. [21] reported that in the reaction with peroxynitrite uric acid undergoes nitrosation, forming a ONO derivative with vasoactive $^{\bullet}NO$-releasing ability. Although these findings were obtained at physiologic pH, we cannot exclude the formation of a similar compound(s) in HS at acidic pH, which in turn could increase NO_2^- after neutralization. Further studies are in progress in our laboratory to characterize the end products of uric acid oxidation in acidified HS.

Importance of uric acid in the gastroesophageal tract

It is reasonable to assume that in the mouth and in the esophageal tract, HS is at neutral pH, so that uric acid is not depleted at least by the action of NO_2^-. When acidification occurs at the gastroesophageal junction and cardia, AH_2, which is secreted into the stomach, is rapidly consumed [2,36] on account of the SCN^--catalyzed nitrosation reaction [32,44]. Under these conditions a burst of $^{\bullet}NO$ is formed, which is in part absorbed by gastric mucosa and in part can form reactive nitrogen species through air autoxidation. The major role of uric acid is the scavenging of $^{\bullet}NO_2$ formed by $^{\bullet}NO$ autoxidation. However, when oxygenated saliva meets the low pH, molecular oxygen is also rapidly consumed by $^{\bullet}NO$ and formation of the nitrating species $^{\bullet}NO_2$ is markedly inhibited, thus slowing down uric acid consumption (Fig. 3). Furthermore, the concerted action of AH_2 and SCN^-, the absorption of $^{\bullet}NO$ by the gastric mucosa, and the antioxidant role of uric acid could protect the stomach from the formation of toxic nitrogen species. Under pathological conditions, such as gastric acid reflux, normoxic HS coupled with a neutral-to-acidic pH switch, could result in a complete consumption of antioxidants and in the formation of nitr(os)ocompounds, thus contributing to the high incidence of tumors in the upper gastrointestinal tract.

Bovine serum albumin and chlorogenic acid spare uric acid through different mechanisms

Our results showed that natural antioxidants representative of a fruit- or a protein-rich meal (chlorogenic acid and BSA, respectively) are able to spare uric acid in acidified HS, albeit through different mechanisms. The antioxidant activity of chlorogenic acid in acidified HS is related to its $^{\bullet}NO_2$-dependent scavenging activity through the formation of *o*-semiquinone radicals and nitroaromatic derivatives [32]. The consequence is that this polyphenol, in addition to sparing uric acid, promotes $^{\bullet}NO$ release from salivary NO_2^- (Fig. 5 and [32]), thus favoring the bioavailability of this molecule at the gastric level. This activity has been reported also for other polyphenols [39,41,62]. BSA is not a $^{\bullet}NO$-promoting compound (Fig. 5) and, in contrast to chlorogenic acid, decreases the $^{\bullet}NO$ released by acidified HS. However, it is able to decrease both NO_2^- and NO_3^- (Fig. 6). It is well known, in fact, that BSA is nitrosated and nitrated in acidic NO_2^- solutions [46–48], a finding that is compatible with the scavenging of both N_2O_3 and $^{\bullet}NO_2$. The lack of $^{\bullet}NO$-promoting activity suggests that the scavenging of these species quickly exhausts the HNO_2. BSA can also scavenge $^{\bullet}NO$ through other mechanisms such as the radical–radical termination reactions with protein-centered radicals (Cys, Met, Tyr, and Trp radicals) formed in the oxidized protein. The finding that the Trp radical can be detected by DBNBS spin trapping indicates that this radical escapes the reaction with $^{\bullet}NO_2/^{\bullet}NO$ but does not exclude the formation of other protein radicals reacting with $^{\bullet}NO_2/^{\bullet}NO$ in the fastest reactions, such as the reaction between Tyr and $^{\bullet}NO_2$ radicals ($k=3.0\times10^{9}\ M^{-1}\ s^{-1}$ [63]) that leads to the formation of nitrotyrosine. As proof that Tyr radicals can be formed, although not EPR detectable, we report the formation of a low level of nitrotyrosine in the reaction of BSA with HS.

It is worth noting that urate partially prevents the formation of BSA nitrotyrosines (Fig. 8, lane 2), an antioxidant action that may be important, though data regarding the toxicity of nitrated amino acids are lacking.

From a nutritional point of view, it is important to emphasize that dietary antioxidants do achieve millimolar concentrations in some foods, so that they are at high concentrations in the gastrointestinal tract during the digestive process. It is therefore evident that foods play a critical role in (i) the nitrate cycle, (ii) $^{\bullet}NO$ homeostasis, and (iii) the formation of toxic nitr(os)ocompounds, so it is not surprising that diet may have a relevant role in the prevention of gastric cancer.

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Eur J Appl Physiol (1997) 76: 566–567

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SHORT COMMUNICATION

J.R. Poortmans · H. Auquier · V. Renaut · A. Durussel
M. Saugy · G.R. Brisson

Effect of short-term creatine supplementation on renal responses in men

Accepted: 17 August 1997

Abstract There is an increasing utilisation of oral creatine (Cr) supplementation among athletes who hope to enhance their performance but it is not known if this ingestion has any detrimental effect on the kidney. Five healthy men ingested either a placebo or 20 g of creatine monohydrate per day for 5 consecutive days. Blood samples and urine collections were analysed for Cr and creatinine (Crn) determination after each experimental session. Total protein and albumin urine excretion rates were also determined. Oral Cr supplementation had a significant incremental impact on arterial content (3.7 fold) and urine excretion rate (90 fold) of this compound. In contrast, arterial and urine Crn values were not affected by the Cr ingestion. The glomerular filtration rate (Crn clearance) and the total protein and albumin excretion rates remained within the normal range. In conclusion, this investigation showed that short-term oral Cr supplementation does not appear to have any detrimental effect on the renal responses of healthy men.

Key words Creatine · creatinine · kidney

Introduction

Creatine (Cr) is a naturally occuring nitrogen compound found primarily in skeletal muscle. Its phosphorylated form plays a pivotal role in energy metabolism by supplying phosphate group to ADP to regenerate ATP. Recently, it has been shown that ingestion of 20–30 g Cr per day for several days can lead to an increase in human skeletal muscle total Cr and phosphorylcreatine (Balsom et al. 1994).

It has been suggested that a nitrogen-rich diet might itself induce chronic renal hyperfiltration and hyperfusion and thereby contribute to the functional and structural deterioration of the kidney (Brenner et al. 1982). Theoretically, the two amino and one carboxyle groups of creatine, as well as its high nitrogen content (32%), could add some strain on the kidney if taken in large excess.

The aim of the present study was therefore to investigate the effect of short-term oral Cr ingestion on the estimated glomerular filtration rate and the protein excretion rate.

Methods

Subjects and protocol

The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine and all subjects gave informed written consent before participating in the study. Five male subjects took part in the present experiment. Their physical characteristics were as follows (mean and SD): age 25.1 ± 2.7 year; weight 82.4 ± 3.3 kg; height 180 ± 3 cm. Each subject was instructed for 5 consecutive days to ingest a total 20 g creatine monohydrate (Ultimate Nutrition Products, Inc, Plainville, USA) per day, distributed in 5 g doses Cr (mixed with 5 g of Gatorade powder, containing sucrose and dextrose) in the early morning, at noon, in the late afternoon and in the evening. Each dose was dissolved in warm water. During this period, the subjects were also instructed to remain on a similar protein diet. Two weeks apart the same protocol was repeated using 5 g doses of placebo (Gatorade powder).

After each five day session, the subjects reported to the laboratory with a 24 h urine collection. Blood samples were drawn from the femoral artery and assayed for Cr and creatinine, Crn (enzymatic colorimetric test PAP from Boehringer Mannheim, Germany). The same enzymatic test was used to determine Cr and Crn in urine. Total protein and albumin in urine were determined by a colorimetric test (Yatzidis 1977) and an immunological method (Metzmann 1985) (Turbiquant Albumin urine Behring), respec-

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J.R. Poortmans (✉) · H. Auquier · V. Renaut
Chimie Physiologique, Institut Supérieur d'Education Physique et de Kinésithérapie, Université Libre de Bruxelles,
Avenue P. Héger, 7B-1000 Bruxelles, Belgium

A. Durussel · M. Saugy
Unité d'Analyse du Dopage,
Institut Universitaire de Médecine Légale,
Lausanne, Suisse

M. Saugy
INRS-Santé, Université du Québec, Pointe Claire, Québec, Canada

Table 1 Plasma and urine contents in placebo and creatine supplementation conditions ($n = 5$), mean ± SD

	Placebo	Creatine supplementation
Creatine		
arterial (μmol · l^{-1})	74.0 ± 69.4	275.3 ± 213.5*
urine (μmol · min^{-1})	7.62 ± 4.57	682.5 ± 266.1*
clearance (ml · min^{-1})	13.5 ± 3.0	365.5 ± 87.0*
Creatinine		
arterial (μmol · l^{-1})	85.7 ± 15.0	92.8 ± 14.1
urine (μmol · min^{-1})	133.5 ± 25.6	142.3 ± 89.5
clearance (ml · min^{-1})	159 ± 19	165 ± 49
Proteins in urine		
total (μg · min^{-1})	39.4 ± 11.6	60.8 ± 12.3
albumin (μg · min^{-1})	8.0 ± 1.9	7.9 ± 0.6

* $P < 0.05$ between placebo and creatine supplementation

tively. The glomerular filtration rate was indirectly estimated by the Crn clearance (ratio of urine excretion to plasma concentration). The same calculation was applied to the Cr clearance.

Statistics

The statistical differences between placebo and Cr supplementation were estimated at a P level < 0.05 (two-tailed) using the Wilcoxon matched-pairs signed-ranks test.

Results

The 5 day period of Cr supplementation increased the arterial content by a mean 3.7 fold while the urine excretion rate increased by a mean 90 fold as compared to the placebo period (Table 1). The average clearance was enhanced 27 fold. On the contrary, neither the arterial Crn level and urine excretion rate nor the estimated glomerular filtration rate (Crn clerance) was affected by the oral Cr supplementation. In addition, Cr ingestion had no detrimental effect on the kidney protein excretion rates (Table 1).

Discussion

It has been reported that excess dietary protein (Tolins et al. 1995) and amino acid loading (Mogensen and Solling 1977; Coppo et al. 1993) are associated with renal hyperfiltration, vasodilation and inhibition of tubular protein reabsorption. These effects can induce proteinuria and progressive kidney impairments.

The present investigation demonstrates several important effects of Cr supplementation. Firstly, it has been observed that oral Cr supplementation had a profound impact on the arterial level of Cr, enhancing the penetration of this compound within the muscle compartment. Secondly, there was a massive urine excretion of the ingested Cr (about 60% of the oral load). When account is taken of the arterial content and the glomerulat filtration rate, this implies an important tubular secretion of Cr, an observation not previously described. Thirdly, the non-enzymatic reaction of muscle Cr to Crn was not modified by the large influx of Cr. It implies that normalization of urinary analyses still can be achieved using Crn values in conditions of Cr loading. Fourthly, the kidney reacted normally to the short-term ingestion of Cr. The estimated glomerular filtration rate and the urine excretion rate of total protein and plasma albumin remained within the normal range of a healthy population (Poortmans and Vanderstraeten 1994). No sign of hyperfiltration were observed under these conditions.

In conclusion, these results demonstrated that 5 consecutive days of oral ingestion of 20 g Cr per day does not appear to have any detrimental effect on the renal responses of healthy men.

Acknowledgements The authors are endebted to Bio Nutri Lab's (Mandelieu, France) which provided the creatine monohydrate and to Isostar Sport Nutrition Foundation (Maastricht, The Netherlands) which partly supported this investigation. We would like also to thank Mrs F. Reding for her efficient technical assistance.

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FNH should stop oral contraceptives.[1-5] However, it is uncertain whether pregnancy should be discouraged or whether surgical treatment is required before or even during pregnancy.[3,5] The few case reports are conflicting. We report 10 women with FNH who became pregnant.

Between 1980 and 1996 191 women presented to our institution in whom FNH was diagnosed. Ultrasound, bolus-enhanced computed tomography, and cholescintigraphy were done. Diagnosis of FNH was made when at least two of the three procedures were unambiguously positive, always including cholescintigraphy. (Patients with equivocal findings and exclusion of haemangioma by labelled red-blood cell scanning or magnetic resonance imaging are considered for surgery because adenoma or even hepatocellular carcinoma cannot be ruled out.) Due to an unclear character of the lesion, tumour-related symptoms, or substantial tumour growth, 109 women had surgery. 82 women with a clear diagnosis of FNH were observed (median age 33 [18–61] years). Routine follow-up included ultrasound every 6 months. Ten of 82 observed women with FNH (12·2%) became pregnant, three patients twice. Median age at the time of tumour diagnosis was 27 (22–32) years. Median tumour size was 10 (4–13) cm. All patients had a history of oral contraception with a median use of 120 (6–168) months. Between the time of diagnosis and delivery a median 33 (2–118) months had gone by. The course of pregnancy was uneventful in 11 of 13 pregnancies. One patient presented with right upper abdominal pain. Another developed vaginal bleeding during the 7th week of pregnancy. No increase in tumour size was observed. Delivery occurred spontaneously in eight cases, by Caesarean section in four, and by forceps in one woman without further problems. All children were alive and healthy. Follow-up after pregnancy and a median of 70 (28–141) months occurred at our institution in seven patients, and in three elsewhere. In eight patients, the tumour size remained the same, and in two a decrease (3 cm and 2·7 cm, respectively) was seen.

During the same period three of 47 (6·4%) women having surgery for hepatocellular adenoma had a complicated pregnancy, before referral to us. They had taken oral contraceptives before getting pregnant. In one case emergency Caesarean had been done during the 34th week for "ablatio placentae" and missing fetal heart sounds. Surgery led to the diagnosis of haemoperitoneum, haematoma, and a tumour in the left lobe of the liver. The second patient had presented with hypovolaemic shock due to a ruptured adenoma 3 days after the expected date of birth. Both patients had still births. The third patient had developed upper abdominal pain 1 week after uneventful caesarean section. Ultrasound and computed tomography revealed intrahepatic bleeding from a liver tumour.

In order to justify observation in patients with FNH a clearcut diagnosis is mandatory, especially since the differential diagnosis between FNH and adenoma may be extremely difficult. In these hypervascularised tumours fine-needle biopsy often has a considerable risk of misdiagnosis and bleeding. Therefore, agreement exists about the need to combine several imaging modalities.[1,4,5] Cholescintigraphy allows visualisation of FNH and highlights the absence of proliferated bile ducts in adenoma. From histological diagnosis of the resection specimen in 62 patients who underwent surgery for solid benign liver tumours, we showed a sensitivity of 82·1%, specificity of 97·1%, positive predictive value of 95·8%, negative predictive value of 84·6%, and accuracy of 90·3%.[4] Therefore, addition of cholescintigraphy to ultrasound and bolus-enhanced computed tomography can lead to a high diagnostic yield, while other authors prefer magnetic resonance imaging.[4] Patients with ambiguous diagnosis should have surgery because hepatocellular adenoma and even carcinoma cannot be ruled out. In cases of observation for FNH, it is reasonable to recommend discontinuation of oral contraceptives until clear data are available. However, there is not enough evidence to advise women against pregnancy or to resect the tumour before pregnancy. Life-threatening rupture during pregnancy may occur in patients with non-operated adenomas, whereas for clearly diagnosed FNH no substantial risks have been found in our cases. It seems to be safe enough to observe tumour size with ultrasound and to favour Caesarean section when there is tumour growth.

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Klinik für Abdominal und Transplantationschirurgie, Medizinische Hochschule Hannover, 30625 Hannover, Germany (A Weimann)

Renal dysfunction accompanying oral creatine supplements

N R Pritchard, P A Kalra

The use of oral creatine supplements as a legitimate means to augment athletic performance in both professional and amateur sportsmen and women is widespread.[1,2] However, the safety of oral creatine has been questioned with the deaths of three American college wrestlers linked to its use. The dosage regimens suggested by the manufacturers are 20 g daily for 3 to 7 days and then 2 to 5 g daily as a maintenance dose. At these doses there have been no reports of severe side-effects other than a mild increase in plasma creatinine that is reversible on discontinuation of the preparation.[3,4] However, we have recently managed a patient in whom substantial renal dysfunction was apparent.

A 25-year-old man with focal segmental glomerulosclerosis presented, 8 years ago, with frequently relapsing steroid-responsive nephrotic syndrome. He had required treatment with cyclosporin for the past 5 years to minimise nephrotic relapses. Plasma cyclosporin concentrations were maintained at 75–125 ng/mL. During this time renal function was normal with serum creatinine values of 100–110 μmol/L, creatinine clearances of 91–141 mL/min, and an isotopic glomerular filtration rate (GFR) of 122 mL min^{-1} 1·73 m^{-2} (documented July, 1993). He was seen in June, 1997, when his serum creatinine was 103 μmol/L and creatinine clearance 93 mL/min. However, when he attended clinic 12 weeks later the serum creatinine was 159 mmol/L and creatinine clearance 61 mL/min. 1 month later (mid-October, 1997) his renal function had deteriorated further (serum creatinine 180 μmol/L and creatinine clearance 54 mL/min). He had remained in good health with no relapse of proteinuria and he was normotensive with a cyclosporin concentration well within the therapeutic range. He denied taking nephrotoxic medication, but he did admit to taking creatine supplements to augment his pre-season soccer training regime. He had started the preparation in mid-August with a loading dose of

5 g three times per day for 1 week and then a maintenance dose of 2 g/day which he had been taking for 7 weeks at the time of the appointment in October. He was advised to stop the creatine, an urgent isotopic GFR was arranged, and a plan to re-biopsy made should his renal function deteriorate further. The GFR 3 days later was 67 mL min^{-1} 1·73 m^{-2}. 1 month after stopping the supplements his plasma creatinine had returned to 128 μmol/L with a creatinine clearance of 115 mL/min.

There is strong circumstantial evidence that creatine was responsible for the deterioration in renal function in this case. Whether this represents tubular damage due to muscle pigment, or is a manifestation of increased creatine production, is unclear. Physicians should be aware that creatine, which is classed as a food-stuff in the UK, is freely available over the counter and is used at excessive doses by some individuals.

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Department of Renal Medicine, Hope Hospital, Salford Royal Hospitals NHS Trust, Salford M6 8HD, UK (N R Pritchard)

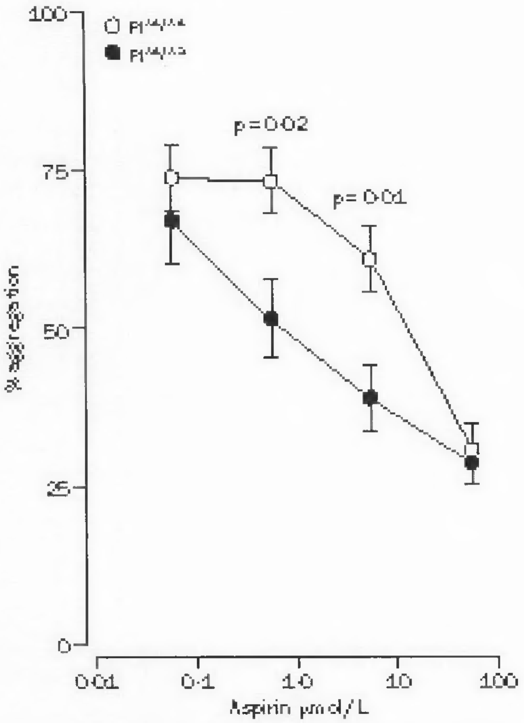
Pl^{A2} polymorphism and efficacy of aspirin

Glen E Cooke, Paul F Bray, Jeanette D Hamlington, Dung M Pham, Pascal J Goldschmidt-Clermont

Aspirin is widely used as an antiplatelet drug to prevent arterial thromboembolic events.[1] Aspirin acetylates irreversibly the enzyme cyclooxygenase (COX-1), and thereby inhibits the production of thromboxane A_2 in platelets. However, the mechanism by which thromboxane A_2 induces the expression of high-affinity receptor molecules for fibrinogen on the surface of platelets (the final common pathway of platelet activation) remains unknown.

We have reported that the Pl^{A2} polymorphism of the GPIIIa subunit of the fibrinogen receptor (GPIIb-IIIa) is a risk factor for coronary heart disease.[2] Feng et al have shown, on 1336 participants in the Framingham Offspring Study that the Pl^{A2} polymorphism increases platelet aggregation in an allele-dependent fashion.[3] While some subsequent studies have found Pl^{A2} to be a risk factor for coronary heart disease,[4] others have not, and in particular, the Physicians' Health Study did not show an association between the Pl^{A2} polymorphism and first myocardial infarction.[5] We hypothesised that a differential sensitivity to aspirin between Pl^{A2}-positive platelets ($Pl^{A1/A2}$ or $Pl^{A2/A2}$) and $Pl^{A1/A1}$ homozygous platelets might explain, at least in part, this discrepancy.

We compared the aggregation of platelets from fifteen $Pl^{A1/A1}$ homozygotes and eleven $Pl^{A1/A2}$ heterozygotes matched for age, race, and gender. Fasting morning blood was obtained without caffeine intake (>12 h) or drug intake (>7 days). Platelet-rich plasma was obtained from citrated blood and the aspirin-sensitive agonist epinephrine was used to induce aggregation. Adenosine diphosphate (1 μmol/L) was added if epinephrine alone was unable to induce 60% or more aggregation. We tested the inhibitory efficacy of aspirin using aspirin concentrations ranging from 0·053 to 53 μmol/L to provide a spectrum that covers concentrations found in patients treated with various dosages of aspirin (0·6 to 14 μmol/L).



Inhibition of aggregation of $Pl^{A1/A1}$ and $Pl^{A1/A2}$ platelets by aspirin

Aggregation in the absence of aspirin was the same in $Pl^{A1/A1}$ and $Pl^{A1/A2}$ platelets. Inhibition of $Pl^{A1/A2}$ platelets with aspirin was greater (figure). The mean aspirin concentration that was required to induce 50% inhibition of the aggregation response (IC_{50} in $Pl^{A1/A1}$ platelets was 22·8 (5·8 SEM) and 2·3 (1·2) μmol/L in $Pl^{A1/A2}$ platelets, indicating a significant (p=0·005) 10-fold, reduction in IC_{50} for aspirin with $Pl^{A1/A2}$ platelets.

We conclude that the mechanism of platelet inhibition by aspirin involves an epitope of the GPIIIa subunit of the fibrinogen receptor that contains the Pl^{A} polymorphism. We suggest that differences in exposure of patients to aspirin at the time of onset of myocardial infarction might account for some of the discrepancies amongst studies on Pl^{A2} as a risk factor for unstable ischaemic coronary events.

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The Heart and Lung Institute (P J Goldschmidt-Clermont), **and Division of Cardiology, Department of Internal Medicine, College of Medicine and Public Health, Ohio State University, Columbus, OH 43210, USA; and Division of Hematology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore**

Clinically Relevant

Long-term oral creatine supplementation does not impair renal function in healthy athletes

JACQUES R. POORTMANS and MARC FRANCAUX

Chimie Physiologique, Institut Supérieur d'Education Physique et de Kinésithérapie, Université Libre de Bruxelles, Brussels, BELGIUM; and Laboratoire de Physiologie, Institut d'Education Physique et de Réadaptation, Université Catholique de Louvain, Louvain-la-Neuve, BELGIUM

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ABSTRACT

POORTMANS, J. R., and M. FRANCAUX. Long-term oral creatine supplementation does not impair renal function in healthy athletes. *Med. Sci. Sports Exerc.*, Vol. 31, No. 8, pp. 1108–1110, 1999. **Purpose:** Oral creatine supplementation is widely used in sportsmen and women. Side effects have been postulated, but no thorough investigations have been conducted to support these assertions. It is important to know whether long-term oral creatine supplementation has any detrimental effects on kidney function in healthy population. **Methods:** Creatinine, urea, and plasma albumin clearances have been determined in oral creatine consumers (10 months to 5 yr) and in a control group. **Results:** There were no statistical differences between the control group and the creatine consumer group for plasma contents and urine excretion rates for creatinine, urea, and albumin. Clearance of these compounds did not differ between the two groups. Thus, glomerular filtration rate, tubular reabsorption, and glomerular membrane permeability were normal in both groups. **Conclusions:** Neither short-term, medium-term, nor long-term oral creatine supplements induce detrimental effects on the kidney of healthy individuals. **Key Words:** ORAL CREATINE SUPPLEMENTATION, KIDNEY FUNCTIONS, ATHLETES

Creatine is a naturally occurring nitrogen compound synthesized from three amino acids and mainly located in skeletal muscle. Its phosphorylated form plays a pivotal role in energy metabolism by supplying phosphate group to ADP to regenerate ATP. Since 1992, a series of studies has suggested that creatine supplementation may be an effective ergogenic substance (6). Feeding 20–30 g creatine per day for several days is a common procedure that can indeed induce an increase in human skeletal muscle total creatine and phosphorylcreatine. Nowadays, creatine supplements are now used worldwide by healthy individuals and athletes to increase their maximal performance and to gain better adaptations during intense training sessions (see reviews 1, 11).

Recently, it has been claimed by Pritchard and Kalra (15) that oral creatine supplements may lead to renal dysfunction. Moreover, press releases have attributed deaths of American wrestlers to creatine supplementation. Both assertions appeared to be overreacting positions. Indeed, we demonstrated that short-term (12) and medium term (13) creatine supplementation in men does not appear to have any detrimental effect on the renal responses of healthy adults. Nevertheless, it has been suggested that a long-term, nitrogen-rich diet might itself induce renal hyperfiltration and thereby contribute to the functional and structural deterioration of the kidney (2). Theoretically, the high nitrogen content (32%) of creatine could add some strain on the kidney if taken in large excess for a long period of time.

To shed light on this specific aspect, we investigated the effect of long-term oral creatine ingestion by highly trained athletes on the estimated glomerular function, urea clearance, and protein excretion rate.

METHODS

Subjects. Eight young men and one women (24 ± 3 yr old), in healthy condition, participated in the present investigation. Being athletes in track and field and volleyball on the national and international levels, they regularly participated in strenuous exercise training (12–18 $h \cdot wk^{-1}$) for the last few years (5–10 yr). Theses subjects have been chosen as voluntary and spontaneous regular consumers of creatine monohydrate (Table 1). Eighty-five male students in physical education and physical therapy, who did not consume

0195-9131/99/3108-1108/0
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Submitted for publication November 1998.
Accepted for publication January 1999.

TABLE 1. Individual consumption of creatine.

	Creatine Supplementation		
Subject	Per day (g)	d·wk^{-1}	Duration (yr)
1	30	7	1
2	2	7	3
3	3	7	2
4	10	7	2
5	10	6	5
6	5 (+ 5 g sucrose)	7	10 months
7	10	7	2.5
8	10 (+ 30 g sucrose)	7	5
9	10	7	3

any ergogenic supplements, served as a control group. The experimental protocol was approved by the Ethics Committee of the Faculties of Medicine of the Université Libre de Bruxelles and of the Université Catholique de Louvain, and all subjects gave informed written consent before participating in the study.

Creatine supplementation. Our highly trained athletes consumed regularly creatine monohydrate of different origins (organic synthesis). The powder was diluted in hot water, and for some individuals an additional load of glucose or maltodextrine was added to the Cr. Individual doses from 1 to 20 g creatine were taken up to 1 to 4 doses per day. Altogether, daily doses from 1 to 80 g·d^{-1} were ingested every single day of the week for a period from 10 months to 5 yr. Side effects were almost absent but muscle cramps (one subject) and headache (one subject) were occasionally reported. However, we do not have evidence that these slight side effects were really related to creatine supplementation.

Blood and urine samples. All subjects reported to the laboratory around 0800 h with a 24-h urine collection. They did not consume creatine during the 16 h before the blood sampling. Sodium merthiolate was added to each urine sample to prevent bacteria growth. Blood samples were drawn from the antecubital vein into EDTA K3 Vacutainer tubes (Becton-Dickinson, Le Pont de Claix, France), the subjects being in a seated position. Plasma was separated by centrifugation at 2200 × *g* during 10 min. All samples were assayed the day of collection.

Biochemical analyses. Creatine and creatinine were determined in plasma and urine using an enzymatic colorimetric test PAP (Boehringer Mannheim, Mannheim, Germany). Creatine was assayed by omission of creatininase in the test tube. Urea was analyzed in both biological fluids by an enzymatic technique (Sigma Diagnostic-Urea Nitrogen No. 640, St. Louis, MO). Albumin in urine was assayed by an immunological method (9) (Turbiquant Albumin urine, Behring). Plasma albumin was determined by a colorimetric assay (5). The urine excretion rates, expressed in units·min^{-1}, were calculated using the 24-h urine output whereas the clearance values for creatine, urea, and albumin were calculated as the ratio of urine excretion rate to plasma concentration.

Statistical analyses. Mann-Whitney's two-sample rank sum test was used to compare any difference between the creatine group and the control group ($P < 0.05$). The results are given as mean ± SEM. All calculations were made using StatView statistical package.

TABLE 2. Mean values (± SEM) of plasma and urine contents of the control and creatine groups.

	Control Group (N = 85)	Creatine Group (N = 9)
Plasma		
Creatine (μmol·L^{-1})	50.3 ± 6.1	62.2 ± 11.0
Creatinine (μmol·L^{-1})	80.4 ± 5.3	72.5 ± 13.3
Urea (mmol·L^{-1})	2.51 ± 0.10	2.45 ± 0.15
Albumin (g·L^{-1})	42.4 ± 1.1	42.5 ± 1.2
Urine		
Output (mL·min^{-1})	0.96 ± 0.11	1.20 ± 0.16
Creatine (μmol·24 h^{-1})	288 ± 96	10,828 ± 3,264*
Creatinine (mmol·24 h^{-1})	16.4 ± 0.6	13.9 ± 1.1
Urea (mmol·24 h^{-1})	188 ± 15	205 ± 22
Albumin (μg·min^{-1})	6.9 ± 1.6	6.3 ± 1.1
Creatine/creatinine (μmol·mmol^{-1})	19.7 ± 2.0	1:108 ± 400*
Clearance		
Creatine (mL·min^{-1})	4.7 ± 1.5	156 ± 55*
Creatinine (mL·min^{-1})	145 ± 8	143 ± 11
Urea (mL·min^{-1})	64 ± 9	86 ± 8
Albumin (μL·min^{-1})	0.19 ± 0.05	0.15 ± 0.03

* $P < 0.001$ between the control group and the creatine supplementation group.

RESULTS

Table 2 reports values obtained in the control and the creatine supplementation groups. There were no statistical differences between the two groups for plasma contents of creatine, creatinine, urea, and albumin. The creatine group had a urine creatine excretion rate and a creatine (μmol)) to creatinine (mmol) ratio far away from the values recorded in the control group. This clearly showed that the first group was a heavy consumer of exogenous creatine supplements. Urine excretion rates for creatinine, urea, and albumin were within the normal range of a healthy young population in both groups. Creatine clearance in the creatine consumer group was similar to the glomerular filtration rate estimated from the creatinine clearance. The other clearance measurements (creatinine, urea, and albumin) did not differ when comparing control and creatine supplement groups. Thus, the glomerular filtration rate (creatinine clearance), tubular reabsorption (urea clearance), and glomerular membrane permeability (albumin clearance) were normal in both groups rejecting any detrimental effect of creatine supplementation on these renal parameters.

DISCUSSION

It has been reported that excess dietary protein (17) and amino acid loading (3,10) are associated with renal hyperfiltration, vasodilation, and inhibition of tubular protein reabsorption. These effects can induce proteinuria and progressive kidney impairments. Very recently, Pritchard and Kalra (15) have proposed that oral creatine supplementation will lead to renal dysfunction. Indeed, these authors reported that their one subject, who had glomerulosclerosis for 8 yr, being treated with the nephrotoxic cyclosporin drug for the past 5 yr, had substantial renal dysfunction after consuming creatine for 7 wk. This report and the widespread use of this

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ergogenic product among athletes meant that the safety of creatine use could be questioned.

Previously we investigated the potential kidney dysfunction induced by oral creatine looking at short-term (5 d) (12) and medium-term (63 d) (13) supplementation. We demonstrated that, under these circumstances, oral creatine supplements did not affect kidney function in healthy individuals. The present report extends these observations by investigating the long-term effects (10 months to 5 yr) of regular oral creatine supplementation. An increase in the urinary creatinine output has been reported during 5 d (7), 36 d (16), and 90 d (4) of creatine supplementation. However, two other reports did not observe any modification of creatinine excretion after 5 d (8) and 10 wk (18) of creatine supplements. Our present study on 10 months to 5 yr creatine supplements confirms these latter observations. The lack of increase in creatinine output under creatine supplementation may be due to the large excess of urine excretion of this dietary supplement. Moreover, Vandenberghe et al. (18) mentioned similar urinary urea output values between creatine and placebo groups as we did. It is also interesting to note that the renal creatine clearance of the supplementation group is similar to their glomerular filtration rate. This observation points out the efficient plasma clearance of excess creatine consumption that is not taken up by the intracellular compartment. The estimated glomerular filtration rate (creatinine) and the urine excretion of plasma albumin remained within the normal range of a healthy young population (14). No sign of hyperfiltration was observed under these conditions.

Thus, the present report confirms our previous investigations that did not postulate any detrimental effect of creatine supplementation by healthy individuals, up to 5 yr. Nevertheless, a more extended survey should be addressed in the near future and it would be worth to stress that those who have suspected renal dysfunction should avoid any creatine supplementation.

We thank the Direction Générale des Sports (Communauté Française de Belgique) for their support as well as Mrs F. Louppe-Reding for her efficient technical help.

Address for correspondence: Jacques R. Poortmans, Chimie Physiologique, ISEPK 168, Université Libre de Bruxelles, 28 avenue Paul Héger, B-1000 Bruxelles, Belgium. E-mail: jrpoortm@ulb.ac.be.

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Sports Med 2000 Sep; 30 (3): 155-170
0112-1642/00/0009-0155/$20.00/0

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Adverse Effects of Creatine Supplementation

Fact or Fiction?

Jacques R. Poortmans[1] and *Marc Francaux*[2]

1 Physiological Chemistry, Higher Institute of Physical Education and Readaptation, Free University of Brussels, Bruxelles, Belgium
2 Institute of Physical Education and Readaptation, Catholic University of Louvain, Louvain-la-Neuve, Belgium

Abstract

The consumption of oral creatine monohydrate has become increasingly common among professional and amateur athletes. Despite numerous publications on the ergogenic effects of this naturally occurring substance, there is little information on the possible adverse effects of this supplement. The objectives of this review are to identify the scientific facts and contrast them with reports in the news media, which have repeatedly emphasised the health risks of creatine supplementation and do not hesitate to draw broad conclusions from individual case reports.

Exogenous creatine supplements are often consumed by athletes in amounts of up to 20 g/day for a few days, followed by 1 to 10 g/day for weeks, months and even years. Usually, consumers do not report any adverse effects, but body mass increases. There are few reports that creatine supplementation has protective effects in heart, muscle and neurological diseases. Gastrointestinal disturbances and muscle cramps have been reported occasionally in healthy individuals, but the effects are anecdotal. Liver and kidney dysfunction have also been suggested on the basis of small changes in markers of organ function and of occasional case reports, but well controlled studies on the adverse effects of exogenous creatine supplementation are almost nonexistent.

We have investigated liver changes during medium term (4 weeks) creatine supplementation in young athletes. None showed any evidence of dysfunction on the basis of serum enzymes and urea production. Short term (5 days), medium term (9 weeks) and long term (up to 5 years) oral creatine supplementation has been studied in small cohorts of athletes whose kidney function was monitored by clearance methods and urine protein excretion rate. We did not find any adverse effects on renal function.

The present review is not intended to reach conclusions on the effect of creatine supplementation on sport performance, but we believe that there is no evidence for deleterious effects in healthy individuals. Nevertheless, idiosyncratic effects may occur when large amounts of an exogenous substance containing an amino group are consumed, with the consequent increased load on the liver and kidneys. Regular monitoring is compulsory to avoid any abnormal reactions during oral creatine supplementation.

For the last 7 years, top athletes first, then sport professionals and amateur sports participants have been using oral creatine supplementation on a regular basis as an ergogenic aid to improve performance. The amount taken of this supplement ranges from a few grams to tens of grams per day for weeks, months or even years. Several reviews have been devoted to the subject, focusing attention on the benefits for performance.[1-9] A recent book by Williams et al.[10] is a highly comprehensive manual for creatine consumers, providing numerous insights on this naturally occurring substance.

On the contrary, there is little information about the adverse effects of creatine supplementation.[11,12] These authors discussed mainly hypothetical effects arising from a small number of scientific publications. Besides this, there has been much invalid information published by newspapers and periodicals. The aim of the present review is to shed light on the facts and to reject several allegations supported by no scientific evidence. Recommendations will be proposed to minimise any health problems arising from creatine supplementation.

1. The Consumers of Creatine

According to the French newspaper *Le Monde*,[13] the American sport community has been under a 'creatine supplement spell'. Up to 75% of the San Francisco 49ers and the Denver Broncos football players are regular consumers of oral creatine. Schneider reported that US Navy Seals (special forces) use several nutritional supplements to increase muscle mass and that creatine is one of the most commonly used supplements.[14] Williams et al.[10] also revealed that over 90% of weightlifters and bodybuilders in the US are regular users of creatine monohydrate. They are male, with an average age of 32 years, have attended or graduated from college and have a substantial income.

The British newspaper *The Independent* surveyed British sportsmen and -women using creatine as an ergogenic aid.[15] More than 360 elite competitors responded to the questionnaire. Nearly 57% had tried creatine and 44% reported using it on a regular basis. Among them were Linford Christie (sprinter, gold medal at the 1992 Barcelona Olympics) and Mark Foster (swimmer, gold medal at the 1998 Commonwealth Games). Among rugby league players and weightlifters, the figure was 100%. However, a number of coaches in Australia and New Zealand who once recommended creatine to their players have stopped doing so for the simple reason that not nearly enough is known about the long term consequences of using it. The British Olympic Association refuses to endorse the substance because of fears over long term effects.

The US Food and Drug Administration (FDA),[13] the Association of Professional Team Physicians[13] and the American College of Sports Medicine[16] concluded that while short term studies look positive for oral creatine supplementation, much more long term research needs to be done before one can issue a verdict on its health status. In doubt, individuals should be left to their free choice and interpretation. However, the medical commission of the International Olympic Committee has decided that creatine is not a prohibited drug but a food supplement; its chairman, Prins Alexandre de Merode, concluded ironically by saying that 'It is like eggs and foie gras, if you take too much you are sick!'. Quite surprisingly, the French Government has decided not to decide. Indeed, a commission of the Ministry of Youth and Sports pointed out that creatine has no legal right to be sold in France. Thus the product cannot be bought on the market nor in pharmacies, and the government does not need to provide any legal judgement on creatine. Meanwhile, they advise against its use.

2. Allegations From the Media

Concern about the deleterious consequences of oral creatine supplementation was initiated in early 1998 when Pritchard and Kalra,[17] 2 British nephrologists, published a paper in *The Lancet* suggesting that there was 'strong circumstantial evidence that creatine was responsible for the deterioration in renal function.' The 25-year-old man mentioned in this study had presented 8 years previously with focal segmental glomerulosclerosis and frequently relapsing corticosteroid-responsive nephrotic syn-

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drome. He had required treatment with cyclosporin, an immunosuppressive drug well known for its nephrotoxicity, for the last 5 years to minimise nephrotic relapses. This man began creatine supplementation for soccer training in mid-August 1997 with a loading dosage of 5g three times daily for 1 week, followed by a maintenance dosage of 2 g/day which he had been taking for 7 weeks. His glomerular filtration rate dropped by 50% but returned to normal values 1 month after stopping the supplements.

Just 3 days after this publication, the French sports newspaper *L'Equipe*[18] reported that creatine is dangerous for the kidneys in any condition. This 'news' was taken up by several European newspapers.[13,19-21] Creatine became the 'champion's Viagra', inducing renal impairment, liver alterations and even death. Indeed, Pritchard and Kalra[17] commented on the cases of 3 American college wrestlers who died. However, we now know from the police investigation that those people did not die from creatine supplements (E. Zambraski, personal communication).

A web site of the FDA[22] gives regular reports of complaints from voluntary consumers of 'nutritionals' or from healthcare professionals. A search of the most recent report (20 October 1998) revealed 32 matches for creatine. The problems reported include dyspnoea, fatigue, grand mal seizure, intracerebral haemorrhage, vomiting, diarrhoea, nervousness and anxiety, polymyositis, myopathy, rhabdomyolysis, severe stomach cramps, deep vein thromboses, atrial fibrillation, cardiac arrhythmia, chest pain and death. Meanwhile, the FDA asks the reader to keep in mind that 'there is no certainty that a reported adverse event can be attributed to a particular product or ingredient. The available information may not be complete enough to make this determination.' In other words, there is no scientific evidence to correlate oral creatine monohydrate supplements with any of these reported adverse effects.

3. Effects of Oral Creatine Supplementation

3.1 Body Mass

One of the effects of oral creatine supplementation regularly mentioned by consumers is an increase in body mass (BM), particularly muscle mass. On scientific grounds, the situation is less clear. Indeed, the average increase in BM reported in the literature amounts to 1 to 2kg or 1 to 2.3% of total BM (table I). However, the effects of short term (<10 days) and medium term (>10 days) supplementation on BM should be distinguished. In fact, short term supplementation is based on a mean of 20 g/day, whereas medium term supplementation generally involves consumption of 2 to 10 g/day. Table I emphasises this difference and its consequences for BM. It may be observed that 68%[23-30,32,41-47] and 70%[45,48,50-54,56,57] of the reported publications showed an increase in total BM for short and medium term supplementation, respectively. Thus, despite the reduced daily dosage of creatine supplements during medium term intake, most of the studies reported an increase in BM. Nevertheless, about 30% of published papers do not report any change in BM, either after short term[31,33-40,55] or medium term[37,39,49,55,58] supplementation.

Two possible reasons may be proposed to explain these discrepancies: the characteristics of the individuals and the supplementation protocol. Sedentary people, physically active individuals and recreational athletes seem to respond equally to oral creatine supplementation. Thus, individuals need not be physically active to increase BM after oral creatine supplementation despite different daily energy intake. Several studies on female volunteers did not show any modification in BM after creatine supplements.[33,34,36,38,55,49] Unfortunately, none of these studies controlled the menstrual cycle of the volunteers. It is currently difficult to postulate a gender dependence, since we do not have any scientific evidence to support those differences. Not all studies have been made using a control group (no training session) in addition to a placebo and creatine supplementation group. We[54] have per-

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Table I. Body mass changes induced by creatine supplementation

Study	Year	Gender	Population	Dosage (g/day)	Number of days	Effect on body mass (%)
Short term (<10 days)						
Balsom et al.[23]	1993	M	Active	24	6	+1.5
Balsom et al.[24]	1993	M	Trained	20	6	+1.2
Greenhaff et al.[25]	1994	M	Active	20	5	+1.0
Stroud et al.[26]	1994	M	Active	20	5	+1.3
Balsom et al.[27]	1995	M	Active	20	6	+1.4
Dawson et al.[28]	1995	M	Active	20	5	+1.0
Green et al.[29]	1996	M	Sedentary	20	5	+1.1
Mujika et al.[30]	1996	M, F	Swimmers	20	5	+1.0
Vandenberghe et al.[31]	1996	F	Active	0.5 g/kg	6	Stable
Becque et al.[32]	1997	M	Weight lifters	20	5	+2.3
Godly & Yates[33]	1997	M, F	Cyclists	20	5	Stable
Grindstaff et al.[34]	1997	M, F	Swimmers	21	9	Stable
Hamilton-Ward et al.[35]	1997	F	Athletes	25	7	Stable
Prevost et al.[36]	1997	M, F	Students	18.8	5	Stable
Stout et al.[37]	1997	M	Football	21	5	Stable
Terrillion et al.[38]	1997	F	Runners	20	5	Stable
Vandenberghe et al.[39]	1997	F	Students	20	4	Stable
Bermon et al.[40]	1998	M, F	Active	20	5	Stable
Maganaris & Maughan[41]	1998	M	Active	11.4	5	+2.3
Oöpik et al.[42]	1998	M	Karatekas	20	5	+1.3
Snow et al.[43]	1998	M	Active	30	5	+1.3
Robinson et al.[44]	1999	M	Active	20	5	+1.4
Volek et al.[45]	1999	M	Trained	25	7	+2.1
Oöpik et al.[46]	1999	M	Wrestlers	20	5	+1.3
Urbanski et al.[47]	1999	M	Active	20	5	+1.0
Medium term (>10 days)						
Earnest et al.[48]	1995	M	Weight lifters	20	14	+1.9
Thompson et al.[49]	1996	F	Swimmers	2	42	Stable
Goldberg & Bechtel[50]	1997	M	Football	3	14	+0.8
Kirksey et al.[51]	1997	M, F	Athletes	0.3 g/kg	42	+2.0
Stout et al.[37]	1997	M	Football	10.5	51	Stable
Vandenberghe et al.[39]	1997	F	Students	5	60	Stable
Volek et al.[52]	1997	M	Trained	3	47	+1.8
Bermon et al.[40]	1998	M, F	Active	3	47	Stable
Kreider et al.[53]	1998	M	Football	16	28	+1.0
Francaux & Poortmans[54]	1999	M	Active	3	63	+2.9
Leenders et al.[55]	1999	M, F	Swimmers	10	14	Stable
Stone et al.[56]	1999	M	Football	8	35	+1.4
Volek et al.[45]	1999	M	Trained	5	77	+6.3
Rawson et al.[57]	1999	M	Old	20	30	+0.6
Francaux et al.[58]	2000	M	Active	21	14	Stable

formed such a comparison in order to be certain that the increase in muscle mass could not be attributed to the effect of training itself (table II). No statistically significant differences in BM were observed in control individuals followed during 9 weeks compared with active people involved in re-

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sistance training for the same period of time. On the contrary, the creatine supplementation group had an increased BM.

The increase in BM could be attributed to the carbohydrate supplements that were added to the creatine ingestion in several studies. Indeed, water binding to muscle and liver glycogen occurs after glucose loading. Green et al.[29,59] and Robinson et al.[44] observed that whole body and muscle creatine retention was increased when large amounts of simple carbohydrates were ingested in conjunction with creatine. However, the conclusions of the 2 studies are not similar. Green et al.[59] reported that creatine loading (20 g/day for 5 days) increased BM by 0.6kg, but when 370g of glucose was added over the course of each day of the study the volunteers gained an additional 1.5kg. On the contrary, Robinson et al.[44] did not observe significant BM change after 5 days of glucose supplementation at 277 g/day, but did report a 1.4% increase (1.0kg) after supplementation with creatine + carbohydrate.

It has been demonstrated that supraphysiological circulating concentrations of insulin, as induced by glucose ingestion, are required to augment muscle creatine accumulation in humans.[60] Consistent with this observation, Willott et al.[61] reported that the highly insulin-sensitive rat soleus muscle had higher rates of creatine uptake than the relatively less insulin-sensitive extensor digitorum longus (EDL) muscle. However, the same authors pointed out that insulin had no direct effect on [^{14}C]-labelled creatine uptake rates at concentrations for which effects are seen on glucose uptake, glycogen synthesis and glycolysis. Moreover, using a competitive inhibitor (β-guanidopropionic acid) of creatine uptake and low extracellular Na^+ concentrations to study the rate of [^{14}C]-labelled creatine uptake in isolated rat muscle, Willott et al.[61] suggested that the rate of creatine uptake is strongly dependent on the extracellular Na^+ concentration, insulin playing only a minor role in the regulation of creatine transfer. Thus, the mechanisms involved in the control of creatine accumulation in skeletal muscles still need to be investigated.

The increase in BM after creatine supplementation has been attributed to fat-free mass.[32,48,51] Nevertheless, this increase in muscle tissue may be due to water retention in the intracellular compartment or to an increase in dry mass. Hultman et al.[62] suggested that the increase in BM during short term creatine feeding is likely to be attributable to body water retention, since they observed a 0.6L decline in urinary volume after ingestion of creatine 20 g/day for 6 days. Furthermore, Ziegenfuss et al.[63] reported a 6.6% increase in thigh skeletal muscle volume and a 2 to 3% increase in total body and intracellular water volume in aerobic and cross-trained men after short term oral creatine feeding. Recently we measured the incorporation of intra- and extracellular water (by bio-impedance) in volunteers receiving creatine supplementation over a period of 9 weeks (table II).[54] The observed increase in BM (2kg) could be attributed partially (55%) to an increase in the body water content and more specifically to an increase in the volume of the intracellular compartment. Nevertheless, the relative volumes of the body water compartments remained constant, and therefore the gain in BM cannot be attributed to water retention, but probably to dry matter growth

Table II. Effect of 9 weeks of creatine supplementation on body composition estimated by bio-impedance in trained volunteers. Values are means ± SD (after Francaux and Poortmans[54])

Variable	Placebo group		Creatine group	
	day 0	day 63	day 0	day 63
Body mass (kg)	71.9 ± 7.3	72.6 ± 6.7	69.8 ± 8.9	71.8 ± 9.0*
Total body water (kg)	39.6 ± 2.5	40.1 ± 2.2	38.0 ± 3.9	39.1 ± 4.1*
Extracellular water (%)	25.9 ± 1.3	26.1 ± 2.0	25.4 ± 1.3	25.5 ± 1.3
Intracellular water (%)	29.5 ± 1.2	29.3 ± 1.3	29.2 ± 1.2	29.3 ± 0.9

* Significantly different ($p < 0.05$) between placebo group and creatine supplementation when comparing the changes between 0 and 63 days.

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accompanied by the normal increase in water volume.

The hypothesis of a dry mass increase during creatine supplementation was introduced in 1972 by Ingwall et al.[64] from *in vitro* experiments on mononucleated muscle cells from breast muscle from 12-day chick embryos. Their experiment demonstrated that skeletal muscle cells formed either *in vitro* or *in vivo* synthesise myosin heavy chain faster when supplied with creatine *in vitro*. The response was apparent within 4 hours after addition of creatine to the culture medium and was concentration dependent over the range of 10 to 100 %mol/L (1.3 to 13 mg/L). Two years later, Ingwall et al.[65] showed that, in cultures of differentiating skeletal muscle, creatine stimulated selectively the rate of synthesis, and not the rate of degradation, of the 2 contractile proteins actin and myosin heavy chain. The same group extended their previous research to isolated hearts from 17- to 21-day fetal mice maintained in organ culture.[66]

More recently, Bessman and Savabi[67] suggested that creatine could induce muscle hypertrophy in adults. They founded their hypothesis on the increased uptake of amino acids by the muscle and thereafter an enhanced biosynthesis of myofibrillar proteins. However, they stated that the concentration of creatine *per se* might not be responsible for the stimulation of protein synthesis seen in physiologically active muscle. It might well be the increased transport of phosphorylcreatine in the intervening space during contraction that makes more energy available for the ribosomes.

Flisinska-Bojanowska[68] conducted an experiment on rats supplemented with creatine (10 mg/day/100g bodyweight). She electrostimulated (50Hz, 10 min/day for 14 days) the gastrocnemius muscle, and fragments of the white and red portions of the muscle were analysed for soluble and myofibrillar proteins. The creatine-supplemented rats had a 16% increase in myofibrillar proteins, specifically in the white portion of the gastrocnemius, when compared with the control group (no creatine, no stimulation). However, when rats were electrostimulated without creatine supplementation, the increase in myofibrillar proteins amounted to 50% in the white portion and 37% in the red portion of the muscle. Creatine supplementation alone did not change the content of the white muscle but increased the myofibrillar proteins in white muscle by 18%. Consequently, these results do not establish a clear beneficial effect of creatine supplements on muscle proteins.

Two further studies support neither the conclusion of Ingwall et al.[65] and Flisinska-Bojanowska,[68] nor the hypothesis of Bessman and Savabi.[67] Brannon et al.[69] investigated the combined effects of creatine supplementation (3.3 mg/g of chow diet) and high intensity run training on the performance capacity and biochemical properties of rodent skeletal muscle. There were no significant changes in either phosphorylcreatine kinase activity or myosin heavy chain isoform distribution following training or supplementation. However, these authors did not give any data on the synthesis of myosin. Fry and Morales[70] reexamined the effects of creatine supplementation on muscle protein synthesis in tissue culture. They could not support the observation of Ingwall et al.[65]

On the contrary, it seems that when rats are depleted of creatine through administration of the analog β-guanidopropionic acid, there is a reduction of muscle myofibrillar proteins and atrophy of fibre II.[71,72] Adam et al.[73,74] investigated running performance and myosin isomers after β-guanidopropionic acid treatment. The drug did not induce any change in running performance, but the myosin isomers appear to be reoriented towards the type I phenotype.

To conclude, there is as yet no evidence that creatine supplementation induces muscle protein synthesis *in vivo*. Experimental protocols are lacking in humans.

Finally, athletes in some disciplines (wrestling, judo, weightlifting, karate, etc.) try to lose BM to compete at an optimal condition below their usual BM. Ööpik et al.[42] asked karate practitioners to reduce their BM by 5% over 5 days while ingesting 20g creatine monohydrate per day during the same period. The creatine group had a smaller reduction

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in BM (–3.3%) compared with the placebo group (–4.3%). Thus, the investigators suggested that the differences in the extent of BM reduction may have been caused by the effect of creatine on this variable. However, there was already an initial difference in BM between the 2 groups, the creatine groups being lighter by 1.7kg. This difference might interfere with the restriction protocol to an unknown extent.

3.2 Muscle Cramps

Anecdotal reports from athletes have claimed that creatine supplementation may induce muscle cramps (see the FDA web site[22]). In a recent unpublished study, we investigated 12 young, healthy, physically active males who received creatine 3 g/day over a period of 28 days. Five athletes experienced at least 1 cramp during sporting activities over the supplementation period (there was no history of cramp for any of these athletes before supplementation). Nevertheless, we have no proof that these cramps are directly related to the creatine supplementation.

The prevalent hypothesis to explain this potential adverse effect was an imbalance in muscle electrolytes. However, Kreider et al.[53] investigated athletes involved in heavy resistance training (5 hours/day) for 28 days. They were supplemented daily with 15.75g of creatine monohydrate. There was no evidence of muscular cramping during resistance training sessions or during performance trials. Along the same lines, Vandenberghe et al.[39] studied sedentary female volunteers who took part in 10 weeks of resistance training with creatine supplementation (20 g/day for 4 days, then 5 g/day up to 10 weeks). No spontaneous adverse effects were reported during the entire duration of the study. Nevertheless, Juhn et al.[75] recently reported muscle cramping in 25% of 52 baseball and football players who were supplemented with 6 to 8 g/day during 5 (baseball) and 3 (football) months.

The anecdotal reports of muscle cramping might be due to the intensity of exercise rather than creatine supplementation. Staying well hydrated could reduce this risk. Moreover, psychological stimulation could cause an individual to exercise over his/her optimal intensity. Further epidemiological studies should be performed to evaluate this potential adverse effect.

3.3 Gastrointestinal Complaints

Even if there are anecdotal reports of gastrointestinal distress (stomach upset, vomiting, diarrhoea) among consumers of oral creatine, these assertions are not supported by real evidence. The scientific literature lacks precise information on this matter. Nevertheless, one report from Vandenberghe et al.[39] stated that one-third of their volunteers (3 of 9) had minor gastrointestinal distress during 3 days of creatine (40 g/day) and caffeine (400 mg/day) supplementation. Also, Juhn et al.[75] reported diarrhoea in 31% of their baseball and football players who were supplemented with 6 to 8g of creatine monohydrate during 5 (baseball) and 3 (football) months. They suggested that this adverse effect may be the result of the unusually high osmotic load imposed on the digestive tract of some study participants. On the contrary, Kreider et al.[53] did not observe any gastrointestinal disturbances among participants in their study of 15.75 g/day. Greenhaff[76] supported this observation in a population ingesting 20 g/day. However, he mentioned that some discomfort can occur if creatine is incompletely dissolved before ingestion.

In conclusion, there is no reason to believe that oral creatine supplementation has any detrimental effect on the gastrointestinal tract.

4. Beneficial Effects in Health and Disease

As well as athletes searching for increased performance, there are individuals who take oral creatine supplements in order to counteract the effects of aging or specific pathological conditions.

4.1 Healthy Individuals

Tissue uptake of creatine into muscles and brain is accomplished by a specific creatine transporter.[77] A recent investigation by Dechent et al.[78] exam-

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ined the extent of cerebral metabolite alterations induced in humans after a single dose (20g) of creatine monohydrate as well as in response to medium term administration (20 g/day) over a period of 4 weeks. The results showed 8.7% increase of total creatine across whole brain areas, with a more specific pronounced effect in the white matter (+11.5%) and thalamus (+14.6%) and no change in water concentration. The oral administration of creatine appears to maintain cellular integrity and normal brain function.

Aging decreases muscle mass, strength and exercise performance.[79] Rawson et al.[80] demonstrated that older men (aged >60 years) supplemented with creatine for 5 days had a slight increase (+0.5kg) in BM, but the increase was still smaller than has been shown to occur in younger individuals. There was a further increase in BM to +0.78kg with creatine supplementation for 30 days.[57] However, there were no statistical differences versus a placebo group who received the same amount of additional carbohydrate load (100 g/day). Nevertheless, the creatine-supplemented group had reduced muscle fatigue.

Bermon et al.[40] followed men and women aged 67 to 80 years who were supplemented with oral creatine monohydrate for 52 days. Some volunteers also underwent strength training for 7 weeks. The results did not show any benefits from oral creatine supplementation in terms of body composition and maximal dynamic strength in the elderly compared with a control group.

However, Smith et al.[81] investigated the effects of creatine supplementation and age on muscle phosphorylcreatine metabolism and performance by using ^{31}P-magnetic resonance spectroscopy. They compared middle-aged (>50 years) and young (<40 years) volunteers consuming 20 to 25g of creatine monohydrate daily for 5 days. Both groups performed a single-leg knee extension exercise. Before the creatine load, the older volunteers had a lower resting muscle phosphorylcreatine concentration compared with the younger volunteers. After exercise, the initial phosphorylcreatine resynthesis was slower in the middle-aged group. After creatine supplementation, resting phosphorylcreatine increased more in the elderly group than the younger group, eliminating the difference in resting muscle observed before the supplementation. Moreover, the phosphorylcreatine resynthesis was increased to a rate similar to that of the younger group after creatine supplementation. Thus, this study indicated that middle-aged individuals had greater improvements in muscle phosphorylcreatine availability, phosphorylcreatine hydrolysis during exercise and initial resynthesis after creatine supplementation.

It seems clear that further studies are needed to validate any beneficial effects of creatine supplements in the elderly.

4.2 Disease States

A few studies have evaluated the benefit of oral creatine supplementation in some pathological conditions. The aim was to provide additional energy stores as phosphorylcreatine to compensate for exertional fatigue and creatine deficit in humans and animals.

4.2.1 Heart Disease

Since total creatine content is at least 20% lower in the diseased human heart, Gordon et al.[82] supplemented patients with chronic heart disease with creatine 20 g/day for 10 days. Before and on the last day of supplementation, the patients underwent exercise on a cycle ergometer. Skeletal muscle biopsies were taken on both occasions. The creatine supplementation did not modify the ejection fraction but increased skeletal muscle phosphorylcreatine and performance as regards both strength and endurance. Andrews et al.[83] extended this previous study to male patients with stable chronic heart failure of at least 3 months duration. The patients received creatine 20 g/day for 5 days. Blood analyses (lactate and ammonium) were assayed before and after creatine supplementation (including a placebo group). The patients exercised the dominant forearm using a hand grip dynamometer (volitional exhaustion at 75% of maximum voluntary contraction). It was observed that creatine supplementation augmented skeletal muscle endurance and attenuated the release of ammonia and lactate in blood.

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4.2.2 Muscle Dystrophies

Fatigue in patients with mitochondrial cytopathies is associated with decreased basal and postactivity muscle phosphorylcreatine. Tarnopolsky et al.[84] supplemented 7 of these patients with creatine (5 g/day for 14 days, then 2 g/day for 7 days). They concluded that creatine supplementation increased the strength of high intensity anaerobic and aerobic type activities in patients with mitochondrial cytopathies but had no apparent effects upon lower intensity aerobic activities.

A recent paper by Pulido et al.[85] extended this previous report to a study of Duchenne muscular dystrophy patients and *mdx* mice. Indeed, it has been reported that human and murine dystrophic muscles have decreased phosphorylcreatine and impaired Ca^{2+} regulation relative to a control group.[86] Primary cell cultures of skeletal cells from normal and *mdx* mice were incubated with creatine (20 mmol/L) for 9 to 11 days. The investigators reported that creatine supplementation enhanced myotube formation and survival when these cells are exposed to high extracellular Ca^{2+} concentration or hypo-osmotic stresses. Based on these observations, Pulido et al.[85] proposed that creatine is a substance with potential therapeutic properties in patients with neuromuscular diseases.

Gyrate atrophy of the choroid and retina is a disease characterised by progressive constriction of visual fields. Atrophy of type II muscle fibres and lower creatine content progress concomitantly with the eye disease. Therefore, Sipilä et al.[87] supplemented patients with creatine 1.5 g/day for a year. The diameters of type II muscle fibres increased by 46% and the visual-field tests showed no further constriction during the therapy.

4.2.3 Neurological Disorders

Excess oral creatine supplementation reaches both muscles and brain, and it has been proposed that creatine may be linked to neuroprotection.

Matthews et al.[88] analysed the role of creatine in Huntington's disease, which involves defects in energy production. They used rats administered 3-nitroproprionic acid to mimic Huntington's disease (intrastriatal inhibition of succinate dehydrogenase). Oral supplementation with 1% creatine dramatically reduced the striatal lesions. The authors are convinced that creatine supplementation may represent a novel therapeutic strategy for neurodegenerative diseases. The same group investigated a transgenic mouse model of amyotrophic lateral sclerosis.[89] The oral administration of 1% creatine in their daily diet improved the survival of the animals (means of 144 *vs* 157 days). Moreover, there was a complete neuroprotection of the neurons in the ventral horn of the supplemented mice as compared with 49% neuronal loss in the control mice. The therapeutic benefit of creatine on survival and motor function of induced amyotrophic lateral sclerosis appeared evident.

Proton magnetic resonance spectroscopy has been used to investigate the effects of oral creatine supplementation in rats with ischaemic events.[90] The animals were supplemented with 2.6g of creatine monohydrate/kg bodyweight per day for 10 days. Despite this huge supplementation, the rats had almost identical dynamics of cerebral lactate and glucose concentrations, even during induced ischaemia. Apparently the elevation of creatine in the brain did not rule out its neuroprotective potential. A similar study was conducted by the same group looking at brain water diffusion in creatine-supplemented rats during transient global ischaemia.[91] They reported a less severe total reduction in apparent diffusion coefficient in creatine-fed rats compared with controls. Hypoxic stress induces a reduction in water diffusion coefficient leading to tissue damage.

Creatine treatment was also applied to a patient with MELAS (myopathy, encephalopathy, lactic acidosis and stroke-like episodes).[92] After 2 weeks of creatine 10 g/day followed by 4 g/day thereafter, the patient reported reduced headache, less weakness and improved work performance.[93] When a child with an inborn error of creatine biosynthesis and total absence of brain creatine was supplemented with oral creatine 400 mg/kg/day,[94,95] the clinical symptoms were markedly attenuated, with a rise of cerebral total creatine of about 50% of the normal value.

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Table III. Effects of creatinine supplementation on serum urea and liver enzymes

Variable	Baseline	Creatine	Dosage (g/day)	Duration (weeks)	Reference
Urea nitrogen (mg/dl)					
	15.8 ± 0.7	13.8 ± 1.0	10	12	Earnest et al.[96]
	15.2 ± 0.1	15.1 ± 0.8	3	9	Poortmans & Francaux[a]
		14.7 ± 0.9	<10	8-260	Poortmans & Francaux[a]
Enzymes (IU/L)					
AST	42.8 ± 22.6	35.7 ± 10.7	3	4	Poortmans & Francaux[a]
	21.3 ± 1.4	22.0 ± 1.2	10	12	Almada et al.[97]
	19.6 ± 3.7	19.1 ± 1.6	3	9	Poortmans & Francaux[a]
	23.1 ± 8.6	26.9 ± 13.0	15.75	8	Kreider et al.[53]
ALT	29.3 ± 15.4	27.0 ± 8.8	3	4	Poortmans & Francaux[a]
	65.8 ± 3.9	70.7 ± 3.7	10	12	Almada et al.[97]
	21.6 ± 2.7	18.7 ± 2.9	3	9	Poortmans & Francaux[a]
	24.1 ± 4.7	28.1 ± 9.8	15.75	8	Kreider et al.[53]
GGT	19.5 ± 5	19.8 ± 7	3	4	Poortmans & Francaux[a]
	24.7 ± 2.8	20.1 ± 1.8	10	12	Almada et al.[97]
	24.7 ± 13.2	25.2 ± 15.8	15.75	8	Kreider et al.[53]
ALP	81.3 ± 18.3	79.0 ± 16.7	3	4	Poortmans & Francaux[a]

a Unpublished data.

ALP = alkaline phosphatase; **ALT** = alanine aminotransferase; **AST** = aspartate aminotransferase; **GGT** = γ-glutamyl transferase; **IU** = international units.

5. Health Risks of Excess Creatine Supplementation

Excessive creatine supplementation may have adverse effects on organs that are involved in its metabolism. The liver synthesises a maximum of 2 g/day of creatine, one-tenth of the regular oral intake used over 5 days by most consumers. Liver enzymes are also indicators of several pathological conditions in liver and striated muscles. Excess creatine has to be excreted by the kidney, and this organ is involved in the homeostasis of the body. Finally, the brain takes up creatine, which could interfere with cerebral metabolism.

5.1 Liver Dysfunction

Despite the allegations published in sports newspapers and periodicals, there is little information on liver metabolism changes induced by oral creatine supplementation. Three publications have reported some data on liver function while consuming creatine supplements.[96-98] These results are summarised in table III, together with data we collected on creatine consumers.

The study of Earnest et al.[96] did not show any significant increase in serum urea and bilirubin throughout the duration of creatine supplementation (20 g/day for 5 days and 10 g/day for 51 days). However, creatine supplementation did increase serum urea by 17% in females. However, the urea levels remained within the range of a normal population. Earnest et al.[96] suggested that long term high-dosage creatine supplementation elicited minimal changes in the markers of hepatic function that were evaluated. Looking at their data and knowing that serum urea is not an accurate representative of liver function, we do not share their conclusion. Indeed, the same group investigated the levels of serum enzymes of liver origin.[97] No changes in enzyme levels were observed during the 8 weeks of supplementation.

Additional information was obtained after oral creatine supplementation in trained individuals (table III). No statistically significant differences were observed throughout the study as far as urea, alkaline phosphatase, aspartate aminotransferase,

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alanine aminotransferase and γ-glutamyltransferase are concerned. Thus, there is no reason to believe that oral creatine supplementation, under the conditions stated in table III, would induce changes in liver function.

A recent report by Duarte et al.[99] indicated that mice supplemented with oral creatine (0.3 g/kg bodyweight for 6 days) had a liver protein content increased by 23%. Among the measured liver enzymes, the authors mentioned that aspartate aminotransferase decreased and alanine aminotransferase tended to increase.

In conclusion, creatine supplementation induces no real modification in liver metabolism.

5.2 Muscle Markers

Phosphorylcreatine kinase (PCK) is commonly used in clinical pathology as a marker of muscle enzyme efflux and thus muscle dysfunction. There are few contradictory reports in the literature related to this specific enzyme under creatine supplementation (table IV). Three publications did not report changes in total plasma PCK after 5 days of creatine supplementation with 20 g/day[98] or 6 g/day,[100] and after 4 to 8 weeks of 10 g/day.[97] However, the latter report showed a slight increase in plasma PCK in male volunteers. Kreider et al.[53] also reported a mild elevation in PCK after 28 days of 15.75 g/day. Nevertheless, it is difficult to get a clear picture of these mild changes in PCK levels, since the athletes were in heavy training, which might itself induce muscle enzyme efflux.

Moreover, plasma PCK exists in 3 different isoforms, namely MM for skeletal muscle, MB for heart and BB for brain. There is no report available that identifies the enzyme isoform that is released from the tissues. Most probably, the slight increase observed in a few reports could be attributed to the skeletal muscle isoform, but precise information is needed to confirm this hypothesis.

5.3 Kidney Impairment

In 1926, Chanutin investigated the fate of creatine when administered to 2 volunteers for 29 and 44 days, with a daily intake of 10 to 20g.[101] The absorption of creatine appeared to be complete. He found an increased creatinine excretion as well as significant positive nitrogen balance. Unfortunately, the excretion was measured for only 1 or 2 days after stopping the administration of creatine. Thus, the carry-over effects on creatinine excretion were not determined. Two years later, Rose et al.[102] reported that after 49 days of feeding creatine 1 g/day, 1 man and 1 woman had a 22 to 25% increase in creatinine excretion. Hyde[103] extended this study, looking at 14 individuals of varying age who received creatine 1 g/day for 4 to 10 weeks. Eight of their volunteers had increased creatinine excretion, whereas 6 did not. Subsequently, Crim et al.[104] gave creatine 0.23 g/day for 9 days and 10 g/day for 10 days, consecutively, to healthy young men. The men were trained on a treadmill (5 days a week) during the 9-day low creatine feeding. Creatinine excretion increased by 10 to 30% during creatine feeding. There was no significant increase in faecal nitrogen during the creatine-feeding period.

More recently, there have been several publications which showed an increase in creatinine excretion

Table IV. Effect of oral creatine supplementation on serum phosphorylcreatine kinase activity

Dosage (g/day)	Duration (days)	Activity (IU/L)		Reference
		baseline	creatine	
10 (M and F)	84	89.3 ± 10.2	97.8 ± 7.8	Almada et al.[97]
10 (M)	56	118.8 ± 18.6	181.3 ± 23.6*	Almada et al.[97]
20	5		No change	Mihic et al.[98]
20	5		No change	Engelhardt et al.[100]
15.75	28	239 ± 106	609 ± 365*	Kreider et al.[53]

* Significantly different ($p < 0.05$) from baseline value.

F = females; **IU** = international units; **M** = males.

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Table V. Effect of creatine supplementation on urea excretion in urine

Dosage (g/day)	Duration (weeks)	Urea excretion (g/24h)		Authors
		baseline	creatine	
5	10	18.82 ± 2.27	19.94 ± 2.10	Vandenberghe et al.[39]
20	1	12.6 ± 2.84	10.33 ± 2.09	Poortmans & Francaux[a]
3	9	12.6 ± 2.84	10.83 ± 6.27	Poortmans & Francaux[a]
<10	8-260		12.3 ± 4.6	Poortmans & Francaux[a]

a Unpublished data.

when individuals were given creatine.[39,40,62,105,106] However, several authors did not observe any statistical changes in creatinine excretion after 5 days,[41,107] 9 weeks[108] or 10 weeks[39] of oral creatine supplementation in trained individuals. Urine output was also measured after creatine supplementation in some studies, and the results are contradictory: either an increase,[40] a stable output[41,108] or a decline in urinary volume.[62] Hultman et al.[62] suggested that the increase in BM during acute creatine loading is likely to be attributable to body water retention. This explanation does not follow from the other studies.

Urea output was also taken into consideration in a few studies. No modification in urea excretion rate was observed after 9 to 10 weeks and 10 months to 5 years of oral creatine supplementation (table V). Therefore, it seems reasonable to say that the liver does not seem to be overproducing urea when healthy individuals ingest creatine in excess.

Creatine at a load of 2 to 20 g/day is totally absorbed by the intestinal tract. However, skeletal muscle cannot take up all this excess creatine and creatine must be excreted in the urine. The excreted creatine represents 40 to 60% of the original load.[41,39,40,107,109]

An early report from Earnest et al.[96] stated that creatine supplementation had minimal changes in the markers of renal function. Unfortunately, they used plasma urea, which does not represent a valuable marker when taken without any urine determination to assess renal function. More troubling, their data did not show any differences in plasma urea for male volunteers and a modest increase (17%) for female volunteers.

The first real investigation (blood and urine values) on renal function during oral creatine supplementation was published in 1998 in the case report described in section 2.[17] This patient, who had pre-existing kidney disease, had taken oral creatine 15 g/day for 1 week and then a maintenance dosage of 2 g/day for 7 weeks. His renal function deteriorated, with a creatinine clearance of 54 ml/min (nor-

Table VI. Effect of oral creatine supplementation on kidney function (excretion rates and clearances)[107,108,110]

Dosage (g/day)	Duration	Baseline	Creatine
Albumin excretion (g/min)			
20	5 days	5.8 ± 0.7	6.9 ± 0.9
3	9 weeks	5.8 ± 0.7	9.0 ± 1.5
<10	8 weeks-5 years		6.3 ± 1.1
Creatine clearance (ml/min)			
20	5 days	3.8 ± 2.5	210 ± 108*
3	9 weeks	3.8 ± 2.5	59 ± 18*
<10	8 weeks-5 years		156 ± 55
Creatinine clearance (ml/min)			
20	5 days	130 ± 16	138 ± 25
3	9 weeks	130 ± 16	143 ± 9
<10	8 weeks-5 years		156 ± 55
Urea clearance (ml/min)			
20	5 days	57 ± 4	50 ± 4
3	9 weeks	57 ± 4	49 ± 8
<10	8 weeks-5 years		86 ± 8
Albumin clearance (μl/min)			
20	5 days	0.14 ± 0.1	0.16 ± 0.02
3	9 weeks	0.14 ± 0.1	0.15 ± 0.02
<10	8 weeks-5 years		0.15 ± 0.03

* Significantly different ($p < 0.05$) from baseline value.

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mal range about 120 to 140 ml/min). Thus, kidney impairment was evident, which was gradually restored after stopping the creatine supplementation.

The only investigations of renal function in healthy individuals who consumed oral creatine supplementation were published quite recently.[107,108,110] We compared the renal clearances of creatinine, urea and albumin in 3 different groups of active individuals who received creatine for 5 days, 9 weeks and up to 5 years with those of control groups. We did not observe statistically significant differences between the control groups and the creatine consumers (table VI). From these experimental protocols we can state that glomerular filtration, tubular reabsorption and glomerular membrane permeability were not affected by oral creatine supplementation using the usual amount (20 g/day for 5 days followed by less than 10 g/day thereafter). Moreover, it is quite easy to determine creatine consumers from the creatine/creatinine ratio in a sample of urine (fig. 1). This ratio is always less than 40 (mg creatine/g creatinine) in nonconsumers and higher than 150 for individuals receiving at least 2g of creatine per day.

Thus, we believe that healthy individuals are not confronted with health risks when consuming reasonable amounts of oral creatine monohydrate. Nevertheless, we urge regular consumers to be tested regularly for excess albumin excretion (>20 μg/min) in urine collected under resting conditions, i.e. after 20 hours of physical inactivity.[111]

However, one case report of interstitial nephritis was published by Koshy et al.[112] in a patient who had taken 20g of creatine daily for 4 weeks. This previously healthy man presented with a 4-day history of nausea, vomiting and bilateral flank pain. The patient was hospitalised with a serum creatinine concentration of 2.3 mg/dl (upper limit of normal range 1.5 mg/dl) and a urine protein excretion of 472 mg/day (upper limit of normal range 150 mg/day). A renal biopsy revealed acute focal interstitial nephritis and acute tubular injury. After stopping the creatine supplements, his renal function subsequently became normal. This is a single case report out of thousands of regular creatine consumers. Nevertheless, it emphasises our recommendation to be tested regularly for urinalysis.

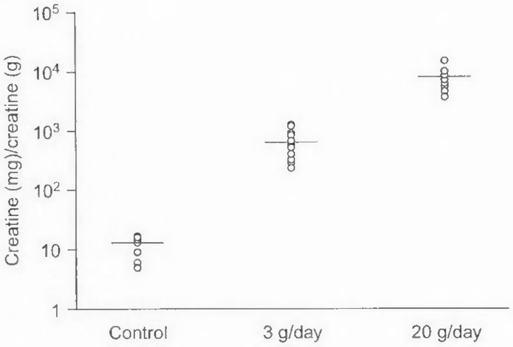

Fig. 1. Urinary excretion of creatine and creatinine, expressed as the ratio of creatine (mg) to creatinine (g) in a urine sample, by control individuals and study participants who ingested creatine monohydrate 3 or 20 g/day.[107,108,110] The horizontal bars represent the geometric means of each population. A clear separation is observed between non-consumers and consumers.

Creatine supplementation has been administered to dialysed patients by Kirschbaum (personal communication) without any adverse effects on blood chemistry.

6. Conclusion and Recommendations

The interest in creatine as an ergogenic aid for athletes has increased over recent years. The purpose of the present study was not to present data and conclusions on the benefit, or the absence of benefit, on sport performance or in various pathological conditions. but to emphasise the potential adverse effects of creatine supplementation in healthy individuals and patients.

It appears that muscle cramping and gastrointestinal distress remain anecdotal. Despite papers and editorials published in the sports media, there is no apparent liver, muscle or kidney dysfunction when healthy individuals consume oral creatine monohydrate at the usual daily amounts (20 g/day for 5 days followed by less than 10 g/day thereafter). Tissues seem to adapt their metabolism by a downregulation of the expression of creatine transporter isoforms, at least in rat skeletal muscle.[113]

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Even if there are no health risks induced by oral creatine supplementation, it is best to remain cautious when this substance is administered on a long term basis. Excess creatine ingestion is still a burden to be eliminated, mostly by the kidney. Regular checkups are advisable to detect any potential dysfunction that may appear in some individuals less able to compensate for any homeostatic imbalance.

Acknowledgements

We are indebted to the Direction Générale des Sports (Communauté Française de Belgique) for their support and to Flamma SpA (Italy) who provided the creatine monohydrate. We also thank Mrs F. Louppe-Reding, Mrs V. Godaert and Mr J. Michotte de Welle for their efficient technical help.

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Correspondence and offprints: Professor *Jacques R. Poortmans*, Chimie Physiologique, CP 168, Institut Supérieur d'Education Physique et de Kinésithérapie, Université Libre de Bruxelles, 28 avenue Paul Héger, B-1000 Bruxelles, Belgium. E-mail: jrpoortm@ulb.ac.be

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